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TITOLO TESI

RECONSTRUCTIVE MICROSURGERY AND TISSUE ENGINEERING IN MUSCULO-SKELETAL ONCOLOGY – INNOVATIVE TECHNIQUES

Presentata da: **Dr.ssa CLAUDIA DI BELLA**

Coordinatore Dottorato **Relatore**

Prof SANDRO GRILLI **Prof PIETRO RUGGIERI**

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1. INTRODUCTION

1.1 SURGERY IN MUSCULO-SKELETAL ONCOLOGY

Thanks to the recent and constant improvements in imaging techniques, chemotherapy protocols, radiotherapy and surgery, the possibility to perform wide tumor resection and subsequent functional reconstruction has become more and more achievable, and now it represents the starting point for the great part of treatment approach in muscolo-skeletal tumor surgery. Compared to 30-40 years ago, ablative surgery (amputation) is reserved to very rare cases, in which the size of the lesion or the neuro-vascular structures involvement influence the treatment choice leading to an absolute impossibility to achieve reconstructive options (1-5). For the majority of the cases, instead, the surgical gold standard is a wide resection followed by reconstruction. Reconstruction techniques are evolving

from purely anatomical to functional for both bone and soft tissues, in some cases with the inclusion of vessels and nerves.

In sarcoma surgery the concept of “margin” is of paramount importance for an adequate treatment. In general, the base principle in the resection of malignant tumors of bone and soft tissues is to obtain “wide” margins, which means excision of the tumor surrounded by a reasonable amount (usually at least 2 cm all around) of healthy tissue with no evidence of tumor (6-9). In fact, any other type of resection, such as “marginal” or “intra-lesional”, is considerate inadequate for sarcoma surgery, as the most likely outcome would be a recurrence of the lesion and possible metastatic spread (10-13). The gold standard, therefore, is the wide excision. This type of resection was considered inadequate in the early ages of sarcoma surgery definitions, while the “radical” excision was considered the best possible treatment. The radical margin, or compartimental, is a resection of the lesion together with the entire compartment in which the lesion is located. It is clear therefore, that in this case the aggressiveness of the surgery is massive and that many times this surgery was synonymous of amputation (Fig.1).

It has been demonstrated over the years that, in terms of overall survival and disease free survival, the outcome of a wide excision are comparable with the outcome of a radical excision, therefore sarcoma surgery has moved towards the concept of “limb sparing surgery” with a decrease of morbidity at the level of the resection (14). Despite these advances in treatment, usually the resection at the level of the

bone or the soft tissue is still quite extensive, and generally there is the necessity for reconstruction in order to guarantee the best possible outcome to the patient. It is therefore extremely important having reconstruction techniques which allow not only a good anatomic and aesthetic coverage of the defect, but even an acceptable functional recovery, especially now that the long term survivorship is definitely longer than in the past.

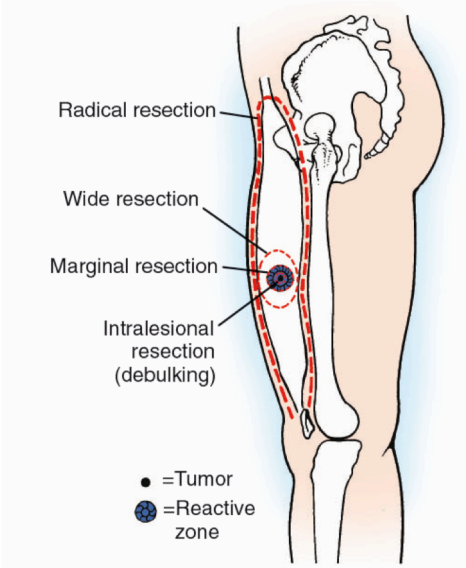

Fig. 1. Enneking classification of margins in soft tissue sarcomas.

- Bone sarcomas

Many type of bone sarcomas are known, and the majority involves young adults. Osteosarcomas (OS) and Ewing sarcomas (ES), in fact, are, among the others, the most common primary bone sarcomas, and the majority of the patients affected are in their 2nd decade of life. On the other hand, Chondrosarcomas (CS), are more commonly diagnosed in adult patients, in general older than 40 years of age. In terms of treatment strategies, while OS and ES usually respond relatively well to chemotherapy (and radiotherapy in the case of ES), CS are not responsive to adjuvant therapies and surgery constitutes the main treatment option. In any case, regardless of the help of chemotherapy or radiotherapy, surgery remains one of the corner stones of treatment, which implies removal of the diseased bone and, therefore, subsequent necessity for reconstruction.

When a sarcoma involves the bone, the main choices for the surgeon are: 1) mega-prosthesis, which can replace the lost bone with adjacent joint, and 2) bone or osteoarticular allograft, which are used when a biologic reconstruction is preferred to a prosthetic one. The advantages of the biologic reconstruction compared to a prosthetic one are to be seen in the view of an improved and longer outcome, especially in the young patient, together with the possibility to re-attach structures such as tendons and ligaments which otherwise would be lost. Therefore some authors believe that this technique, if properly performed and in the absence of complications, could allow a

superior quality of the reconstruction compared to the prosthetic technique (15, 16).

It is well known, however, that the risks associated with a biologic reconstruction are generally superior compared to a prosthetic one, and in fact many complications can occur and are understandably discouraging, especially because they increase with the increase of the follow-up (16-18). It is therefore absolutely necessary an improvement of this technique, especially in the possible strategies that allow a re-vitalization of the allograft. In this optic, tissue engineering could represent the ideal solution, and researchers around the world are working to define the best possible strategy to obtain the perfect and most durable biologic reconstruction.

- Soft tissues sarcomas

Soft tissue sarcomas (STS) are malignant lesions arising in the muscle or subcutaneous tissue of the body, or more rarely in a nerve or in the joint space. As it happens for bone sarcomas, there is a wide variety of different pathologic types of sarcomas, which are defined on the base of the type of cells that they express the most from the histologic point of view; the most common STS are liposarcoma, Malignant Fibrous Histocytoma (MFH), Leyomiosarcoma, Pleyomorphic sarcoma, Schwannoma. All these lesions are more commonly diagnosed in adult patients, in general after the 4th decade

of life, and the vast majority of them responds relatively well to adjuvant radiotherapy. From the surgical point of view, depending on where the lesion is, what is its size and which compartment of the body is involved, the excision could be quite extensive and could involve large part of the skin and/or the subcutaneous tissue. In the majority of the cases the surgery is "conservative" enough to guarantee a good primary closure of the wound. In some cases, however, there is the necessity of cutaneous or musculo-cutaneous flap in order to be able to close the wound, and this happens when the lesion oblige the surgeon to excise big part of the superficial tissue with the impossibility of a primary wound closure.

Thanks to plastic surgery techniques, it is possible now to excise extremely big soft tissue lesions without the necessity to amputate the limb, as long as the main neuro-vascular bundle is not involved and can be safely dissected.

The use of plastic flaps, however, is able to guarantee only a coverage of the defect left after the tumor excision, without the possibility of regain the function of the lost muscle(s). The use and advantages of innovative plastic reconstruction techniques is the goal of the second part of this study.

1.2 SURGICAL RECONSTRUCTION AFTER BONE SARCOMA RESECTION

After bone loss for sarcoma resection, there is the necessity to replace the defect. To do this, the surgeon can choose between a prosthetic reconstruction and a biologic reconstruction.

- PROSTHETIC BONE RECONSTRUCTIONS

After massive bone resections in limb sparing surgery, there is clearly the necessity for anatomic and functional reconstruction. When the lesion, and therefore the resection, involves an area of the bone particularly close to, or at the level of the joint, the gold standard reconstruction technique is the use of a mega-prosthesis (19-23). These type of special prosthesis are particularly useful in tumor surgery, because they not only replace the joint, as a normal orthopaedic prosthesis does in case of, for example, osteoarthritis, but they allow the replacement of the resected bone as well (21, 24, 25). Sometimes these prosthesis can reconstruct the entire length of the bone, as in the case of a total femur replacement, in which both the knee and the hip joint are replaced together with the entire femur (22, 26-28). Mega-prosthesis are the best possible strategy when there is the necessity to sacrifice a joint, because they allow a hypothetical

return to function when the muscles that govern the replaced joint are still in place and functional (29-33).

The main quality of mega-prosthetis, together with their ability to replace the missing bone and joint, is that they guarantee a relatively long functional life and the possibility for the patient to start mobilizing in the immediate days after the surgery (29, 31, 32).

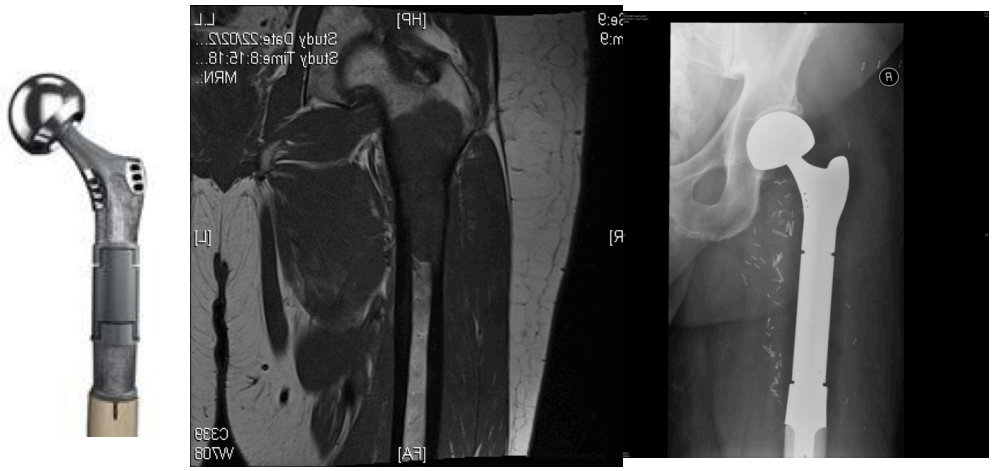

Fig. 2 Example of bone sarcoma located to the proximal femur, treated with proximal femur resection and reconstruction with proximal femur mega-prosthesis

Despite the numerous advantages in the use of mega-prosthesis, there are many conditions that can impair their function (25) and

limit their success: 1) The risk of infection is very high, and in particular is much higher compared to joint replacement prosthesis for arthritis (34). This is because the bone-cartilage resection is much bigger, the length of the surgery is longer with longer exposure of the wound, usually patients are immune-compromised due to neo-adjuvant chemotherapy or radiotherapy and, finally, the soft tissue coverage of the prosthesis might be insufficient. In a recent study, infection has been proven to be the most common mode of failure for this type of prosthesis (35); 2) The achievement of complete function is generally somehow limited due to the loss of muscle and the difficulty and sometimes impossibility to re-attach musculo-tendinous insertions into the prosthesis (25, 36); 3) In the young patient, the longevity of the prosthesis is usually insufficient, with the need of multiple revisions and loss of bone stock, which increases the morbidity of this treatment together with the risk of subsequent infection; 4) Aseptic loosening has been considered one of the most common cause of failure in both cemented and non cemented implants, and is associated with reduction of the longevity of the reconstruction; at the same time, this complication limits the possible surgical options for revision because of the decrease of available bone stock (35).

Taking into account all these aspects, it is clear how an improvement in the current techniques appears necessary, because there is the absolute need to improve the clinical and functional

outcome of our reconstructions and, at the same time, the quality of life of these already proven patients.

- BIOLOGIC BONE RECONSTRUCTIONS

When the bone segment involved by the tumor is relatively far from the articular joint, a biologic reconstruction can be taken in consideration. The reason for the relative exclusion criteria in case the joint is involved refers to the early degeneration of the cartilage, which leads to early osteoarthritis and consequent necessity for relatively early new joint replacement intervention (18, 37-40). In some occasion, however, especially when the patient is very young (usually up to the adolescence), a biologic reconstruction can be attempted even to replace a joint, with the hope that re-vascularization and synovial fluid re-constitution would be able to give enough nutrients to the replaced cartilage to survive (41, 42).

The most common material used for biologic reconstruction is allogenic bone (or bone allograft). This type of tissue is provided by the so-called “bone banks”, which are institutes that store bones harvested from organs donors. One of the most important qualities of bone and cartilage allografts compared to other tissues or organs is that there is no need for immune-suppressive therapy for the recipient, because this tissue does not have enough surface antigens to provoke and immune reaction (43).

The main advantages in the use of bone allografts are to be seen in the possibility to replace significant bone and sometimes osteo-chondral defects maintaining all the main bone-tendon and bone-ligament attachments, therefore allowing a better and more anatomical function compared to a mega-prosthesis (44, 45). If satisfactory results are obtained, the reconstruction can last for many years with a great functional advance for the patient. (Fig.3). Potentially the implication of a biologic reconstruction, therefore, is extremely attractive, however there are still many reasons why the use of allograft is limited to selected cases and maintain the use of prosthesis as a gold standard in the majority of the cases.

The main disadvantages in the use of a biologic reconstruction are:

1. Risk of non-union or pseudo-arthrosis (46). The integration between the host bone and the allograft is one of the weak points of this technique. The allograft, in fact, is a structure that is constituted by dead bone/cartilage, therefore the integration process occurs only in one direction, from the host to the graft, and depends only on the quality of the host bone. The process ideally should, with time, replace the entire allograft with host bone, but in reality the integration between the 2 structures involves only few millimiters of the implant, while the rest of the allograft remains constituted by non-vital bone (47-49). As a consequence of this limited and slow integration process, the rest from any activity has to be prolonged for at least 3 months, and sometimes

patients require up to 12 months before they can start weight bear on the operated limb (50, 51).

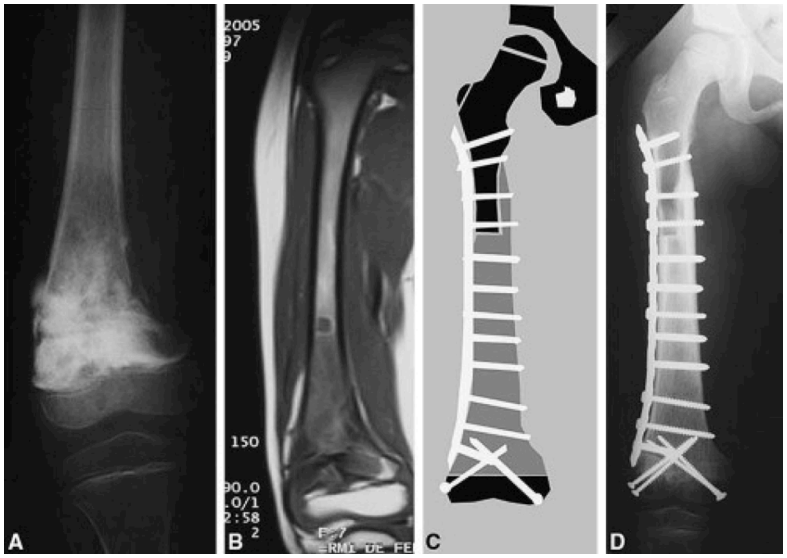

Fig. 3 Case of a 6-year-old boy with a diagnosis of osteosarcoma in whom a transepiphyseal resection and reconstruction with an intercalary allograft was performed. (A) The anteroposterior radiograph of the knee after neoadjuvant chemotherapy shows the osteosarcoma compromises the medial cortex with a varus deformity. (B) A coronal T1-weighted MRI image shows the metaphyseal and diaphyseal extension. (C) A schematic drawing shows the preoperative planning of the reconstruction. (D) The anteroposterior radiograph shows the intercalary allograft after 2 years of followup. (REF: Muscolo DL, Ayerza MA, Aponte-Tinao L, et al. Allograft reconstruction after sarcoma resection in children younger than 10 years old. Clin Orthop Relat Res. 2008;466:1856-1862, with kind permission from Springer Science+Business Media.)

2. Risk of infection (52). The causes are the same present for prosthesis, such as the long length of the surgery and the fact that the patient is usually immune-compromised. To this the

aspect of the allograft biology needs to be added. The allograft, in fact, being for the main part non-vascularized and non-vital, can represent an ideal environment for micro-organisms.

3. Risk of fracture (53). This risk is again due to the avascularity and non vitality of the bone, which cannot undergo to the usual mechanism of micro-cracks and remodelling of normal bone. In this situation, in fact, the micro-cracks will occur without repair and with time this will lead to a proper fracture.
4. Risk of early osteoarthritis. In the case of osteo-articular allografts, cartilage remains constituted by entrapped and not-vital chondrocytes, which cannot receive any nutrient, nor from the synovial fluid nor from the subchondral bone. This will inevitably lead to degenerative changes and, in the long term, early onset of osteo-arthritis (54, 55).

When all these possible risks of failure are analysed together the fear on the use of massive allografts is understandable. However, with a closer analysis, it is clear how every single element of risk is strictly correlated to the vitality of the graft: an allograft that cannot be incorporated and, therefore, remains constituted by dead bone, will have all of these risks which, moreover, will increase overtime. On the other hand, if a strategy to guarantee early re-vascularization and re-vitalization of the graft is found, all these risks will decrease and potentially disappear, and this will greatly improve the treatment

options that can be offered to patients with bone tumors. It is evident, therefore, how a technique to allow bone allograft re-vitalization could potentially represent an ideal solution for these patients and a valid option to prosthetic replacements.

1.3 PLASTIC SURGERY AND RECONSTRUCTIVE MICROSURGERY IN MUSCULO-SKELETAL ONCOLOGY

When the tumor involves the soft tissues (muscles, ligaments and tendons, adipose tissue, skin), there are mainly two strategies of wound closure after the resection: 1. Primary closure. 2. Plastic reconstruction, from simple skin grafts to more complex techniques such as free or rotational flaps. The indications depend on the amount of tissue that needs to be replaced, the available skin coverage and the necessity or not to cover prosthesis or allografts.

- SOFT TISSUE RECONSTRUCTIONS

In many cases, when the tumor involves muscles or subcutaneous tissue, there is the chance for the orthopaedic surgeon to suture the wound without the need of the plastic surgeon. This happens only when the resection, being wide, is still limited and allows a satisfactory closure of the skin flaps without excessive tension. As a

general rule, the main goal of the wound closure, is to re-suture muscular groups with the same function or, in general, without an opposition function. In many cases, a substantial portion of the muscle where the lesion was has been resected, leaving the surgeon with a small fragment of tissue that helps only as “gap filler”. Usually, the function of the limb is severely impaired, because there is no reconstruction of the functional units lost with the resection, and other muscles, with time, tend to hypertrophy to partially replace the lost function. In addition to this, aesthetic is also compromised because of asymmetry with the contra-lateral side and “depression” at the level of the resection. In many cases this is only a minimal discomfort for the patient, but sometimes, in areas such as the adductor compartment of the thigh, following the resection an abundant seromatous/haematic collection follows (56).

Plastic reconstructive surgery is the solution when the defect is too big to be covered with primary closure; plastic techniques allow a more accurate anatomic reconstruction, usually with a better accepted aesthetic results (57-59). Plastic reconstructive techniques are numerous and depend on the size of the defect, the area that needs to be replaced, the amount of muscle that has to be replaced, and the quality of the skin. In the case of big sarcoma resections, typically a free or rotational muscolo-cutaneous flap is the surgeon’s choice because it provides adequate coverage despite the bulkiness that sometimes remains at the level of the flap (60).

- FREE FLAPS IN SARCOMA SURGERY

The use of free or rotational flaps is an extremely valuable resource in soft tissue reconstruction; in the most recent years, the use of this type of flaps is not anymore restricted to the cases in which there is the necessity to cover the prosthetic or biologic bone reconstruction or in cases in which the primary closure cannot be achieved. In fact, the use of flaps has became more and more common in sarcoma reconstruction, with particular indications in which, despite the possibility of a primary closure, a flap is preferred because of the decrease of post-operative complications. This is the case, for example, of the lesions in the adductor compartment of the thigh. In this area, in fact, a wide excision of the lesion often ends with the neurovascular bundle isolated and dissected free of the lesion and of the muscles; even when the primary closure is achievable, the large empty space and the loss of the lymphatic drainage together with a big number of the venous branches, brings to the formaton of a large haematoma/seroma which cannot be promptly re-absorbed by the body with a severe increased rick of post-operative infection (56). Furthermore, the radiation therapy given preoperatively will modify the quality of the surrounding tissues, with a high prevalence of fibrosis and a further decrease of the ability to re-absorb the collection. In a second time, this collection eventually tends to organize and becomes harder, and the differential diagnosis with a

recurrence results difficult, with an understandable stress for the patient and the clinician. Being able to avoid this post-operative trend is of paramount importance, therefore very often now a free or transposed flap is the reconstruction of choice when the lesion is localized in that area or in areas with similar outcomes. Musculo-cutaneous flaps, in fact, not only have the ability to fill the space with autogenous tissue, but, being vascularized, can guarantee the survival of the tissue and the decrease of the morbidities (Fig. 4)

Various techniques are available to the plastic surgeon when there is the need to fill the empty space following tumor resection. In general, a muscolo-cutaneous flap is the most common method used in this type of reconstruction (61). The flap can be rotated from an adjacent area (as, for example, in the gastrocnemius rotation to cover the proximal tibia mega-prosthesis), or can be removed from a different part of the body and positioned where necessary (free flap). This last scenario is one of the most common. The most used free flaps are: parascapular, gracilis and TRAM (transverse rectus abdominis muscle) (62).

To survive, the free flaps need to be secured to an artero-vascular pedicle with micro-anastomosis at the level of the vascular bundle in the recipient area, through termino-terminal anastomosis, or, when a major vessel is involved, through a termino-lateral anastomosis. The ability of the micro-surgeon to perform these sutures is extremely important for the success of the procedure, because if these fail the flap will not survive and a new procedure will be necessary.

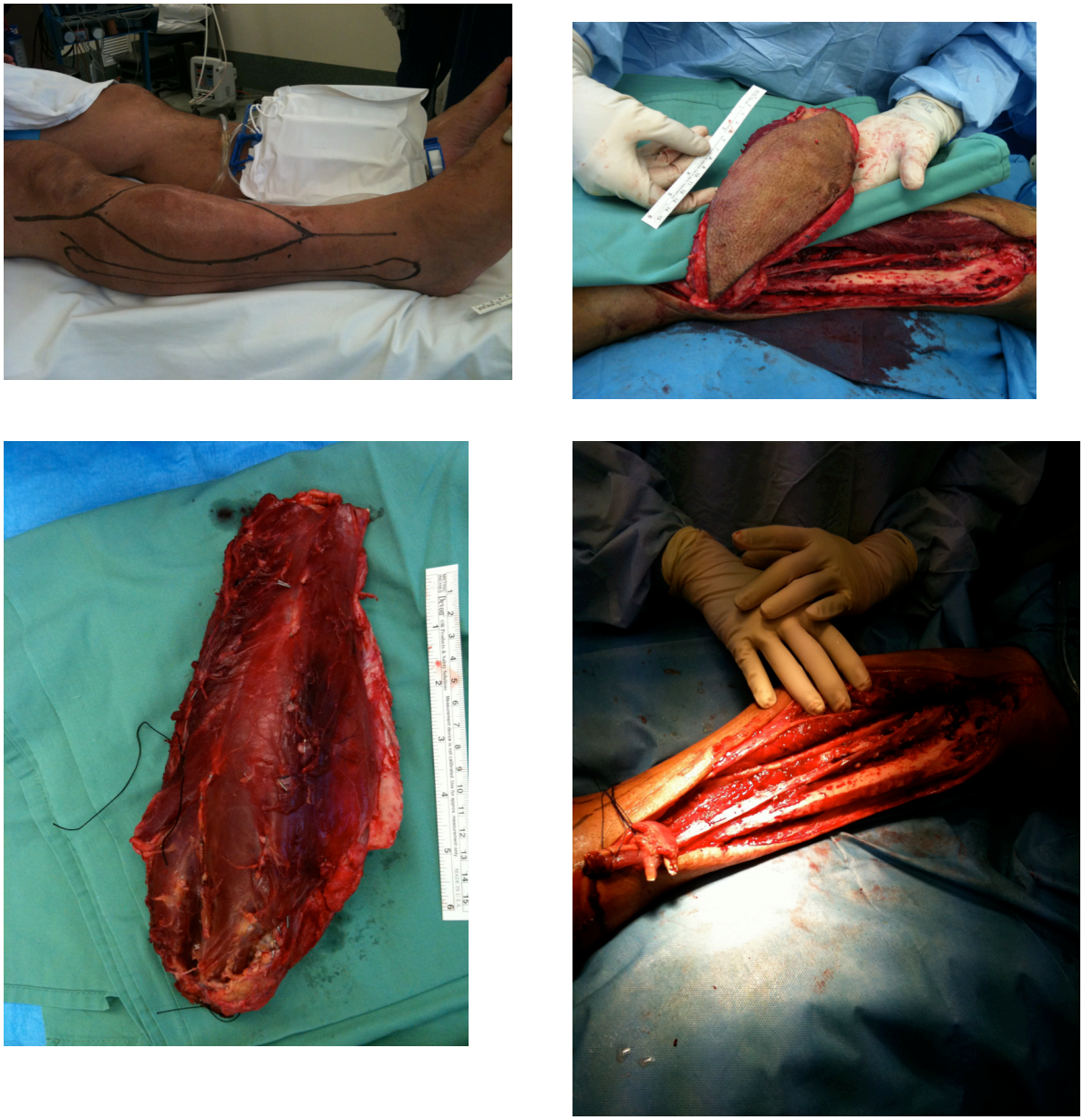

Fig. 4 Example of post radiotherapy soft tissue sarcoma in a 42 yo man located in the antero-lateral compartment of the lower leg. The wide resection lead to important muscle sacrifice, with extensive soft tissue loss and bone exposure. Note in quadrant 3 the inner margin of the resection, showing the healthy muscle covering the lesion.

- VASCULARIZED FLAPS AND THE ROLE OF INNERVATED RECONSTRUCTIONS

Musculo-cutaneous flaps are therefore a very important resource for the reconstruction, however the implanted muscle is not functional. When a large soft tissue lesion is excised, together with the tumor a large amount of muscle is lost because has been replaced by the sarcoma or because its resection is required to obtain wide margins. This functional unit cannot be reconstructed with a traditional flap, because the muscle that is re-implanted will be alive but not functional. In many cases this represents a minor problem, because other fellow muscles can partially replace the lost function, but when the major component of a joint mechanism is lost, such as, for example, the entire extensor mechanism of the knee, then the possibility of having a functional reconstruction becomes very appealing In the case of the anterior compartment of the thigh, in fact, a wide excision usually involves the complete loss of the knee extensors (quadriceps) which act as knee stabilizers as well; in this situation the patient is not able to put weight on the affected limb without an appropriate support which stabilizes the knee in extension.

It is clear, therefore, how important is to find new advanced techniques for soft tissue reconstructions which allow not only the coverage of the defect with viable tissue, but even the ability to re-gain

at least partially the lost function in order to achieve the best possible outcome.

Recently, advances in plastic reconstruction techniques lead to the development of a new procedure that will permit not only the anatomical filling of the defect with vital tissue, but the function of this tissue in synergy with the other muscles around. The innervated free flap, in fact, is a free flap that is harvested not only with its vascular pedicle, but even with its nerve pedicle for motor function. Therefore, once positioned, these flaps can be connected to the nerve that has been resected during the tumor procedure and, hence, thanks to the reinsertion into the remaining muscle or directly into the bone/prosthesis, these flaps recover their contractile mechanism and can improve the patient functional outcome.

Innervated flaps have been attempted for the first time in animal studies by Tamai in 1970 (63), where the feasibility of free neurovascular muscle transplantation has been showed. These studies began the era of microvascular flap innovation, and since then few other reports described this technique in clinical situations. In orthopaedic surgery, innervated flaps has been used for sensate reconstruction of the heel and the ankle, in which the medial plantar flap has been used (64), and has been shown to be a versatile flap capable of providing immediate sensate coverage for heel and ankle defects. Rigorous neurologic testing has shown that when carefully dissected with preservation of its sensory branch, normal sensation can be readily maintained throughout the flap. In general, sensate

innervated flaps has been used for foot and hand reconstructions with different results (65, 66), and it has been demonstrated that while reinnervating a muscle flap with a sensory nerve will permit reinnervation of the muscle and the overlying skin, it is still unclear whether this provides a superior result in durability and, in the case of the foot, gait (67).

On the other hand, there is not a wide literature with regards to motor innervated flaps for the restoration of a muscle function rather than sensation. In sarcoma surgery, this would be the main goal after the resection of the mass, especially when it involves muscular groups important for ambulation, such as the extensor mechanism of the knee or the hip, or when it involves the upper limb and the daily living activities. The first case reports have been presented started form the mid 90s, describing the use of free muscle transplantation for the reconstruction of the anterior compartment of the thigh (68), and more recently the surgical technique has been throughoutly described (69). Starting from the reconstruction of the quadriceps, more muscular functions have been proposed to be restored with this technique, and in 1999 Ihara described the use of innervated flaps for reconstruction after resection of soft tissue sarcomas achieving very good aesthetic and functional results (70, 71). This procedure, although complex and time expensive, is very promising in the view of improving as much as possible the outcome in limb salvage procedures, and is the focus of the second part of this thesis.

1.4 TISSUE ENGINEERING IN ORTHOPAEDICS

Tissue Engineering is a branch of Regenerative Medicine that involves the creation of an organ or a tissue in laboratory, and its subsequent transplant in vivo.

Musculoskeletal tissues, such as bone and cartilage, are since many years the focus of a very wide number of studies in tissue engineering, because they represent a relatively "easy" tissue to reproduce in laboratory and with a very large clinical demand.

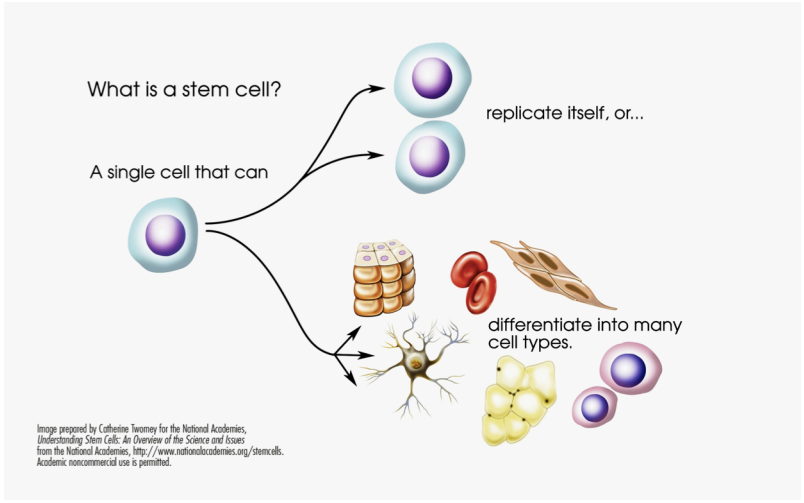

Fig. 5 Representation of Stem Cells and their possibilities

The cells derived from the bone marrow with proliferative capacity toward the mesenchymal lineage are called Mesenchymal Stem Cells (MSC) and are considered multipotent cells. Multipotency represents the characteristic of these cells to reproduce themselves and, at the same time, to differentiate toward a selective lineage for different tissue and organs (Fig. 5).

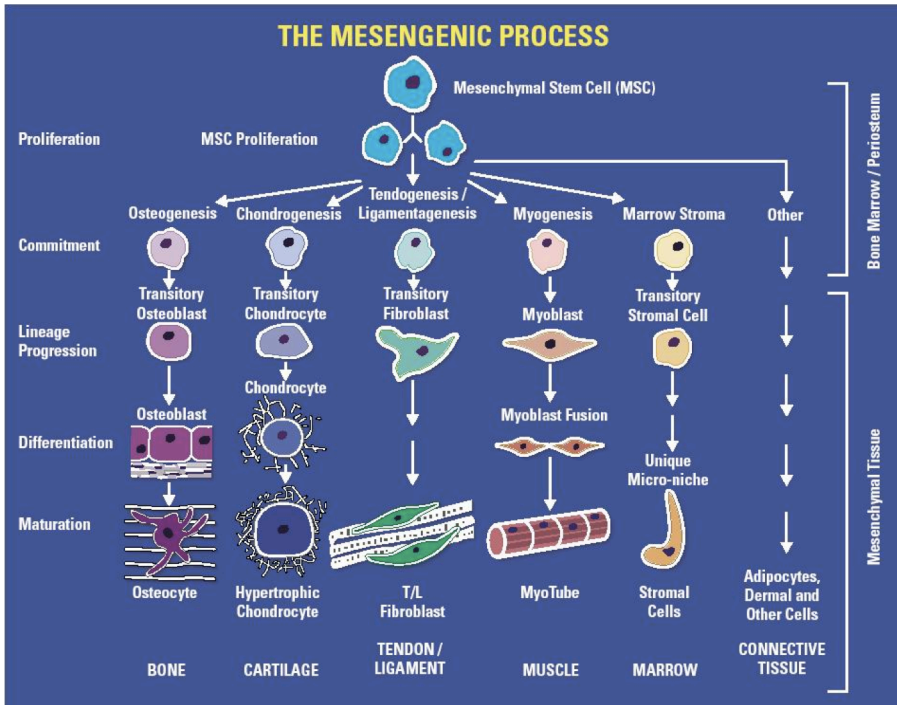

Fig 6. Differentiation abilities of MSC

By using special culture media with selected growth factors, these cells have the ability to grow and to become more and more differentiated toward the chosen lineage (Fig. 6).

When osteogenic growth factors are chosen, for example, these cells after few passages in culture start to loose their proliferative capacity and gain the ability to differentiate themselves, and therefore become progressively osteoblasts and finally osteocytes with the capacity to produce bone matrix and mineralize. With this progression, the more the cells are moving towards the end of the differentiating line, the more they lose the ability to replicate themselves. On the other hand, when the cells are in a very undifferentiated status, they theoretically can replicate with no end. Growth factors used in laboratory to promote differentiation are chemical constituent that act by miming the function of autologous growth factors; it is however possible to use directly the autologous growth factors when they are collected from the cells that biologically constitutes their origin and their reservoir.

With respect to osteogeneic tissue, the most used growth factors are the ones harvested from platelets, such as Platelet Derived Growth Factor (PDGF) and Transforming Growth Factor β (TGF-β). While platelets are obtained from the peripheral blood and subsequent centrifugation and exclusion of the red component, to obtain Mesenchymal Stem Cells (MSC) a bone marrow aspiration is necessary because they are one of the component of the red marrow.

It is well demonstrated now that MSC are found not only in the bone marrow, but potentially in every tissue, because it is postulated that in each tissue there is a reservoir of progenitor cells (72-74). This has been specifically demonstrated in adipose tissue, which currently represents a widely used source of MSC (called Adipose Derived Stem Cells, ADSC) especially because of the simplicity of the harvest and the consistency of the results (75-77).

Stem cells, as specified before, regardless of their origin, when cultured in vitro with osteogenic media, can differentiate and transform themselves in cells forming bone (78, 79). To make this process, growth factors and cells are not enough; in fact, there is the necessity of a third element that allows cells to growth in a 3D construct, which represents the in vivo structure that they will need to reproduce (80). The third element is a scaffold, which act as a tridimensional support for the proliferation and, at the same time, direct the differentiation toward the correct line. Many materials can be used as scaffolds, going from simple organic fluid such as collagen, to more complex 3D structures constructed with highly advanced technology, such as nano-materials. With respect to bone, materials that can possibly be used as scaffolds need to have specific characteristics such as: a strength modulus similar to bone, adequate porosity, biocompatibility and the ability to function as osteoconductive and osteoinductive material. Some of the most used scaffolds used in laboratory are Hydroxyapatyte, Ceramic, Poly-α-hydroesters and other natural polymers as collagen and Chitosan.

Overall, however, the best possible scaffold for bone reconstruction is bone itself, which has already the required 3D structure and can be demineralized to further stimulate the osteogenesis.

Stem Cells, Growth Factors and Scaffold are the three fundamental elements in every tissue-engineering project. Stem cells are the primary elements from which the new tissue will form, Growth Factors are the "fuel" that stimulate these cells into the desired direction and differentiation, and the Scaffold is the 3D structure in which the cells can growth (Fig 7 and 8).

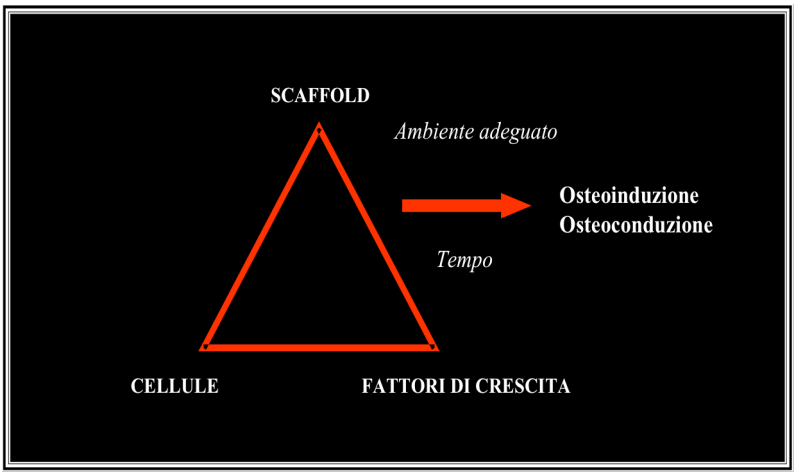

Fig. 7 Scaffold, Cells and Growth Factors are the 3 fundamental element in every tissue engineering process. With an adequate substrate and environment the final outcome is the achievement of the two most important processes in bone regeneration: osteo-induction and osteo-conduction

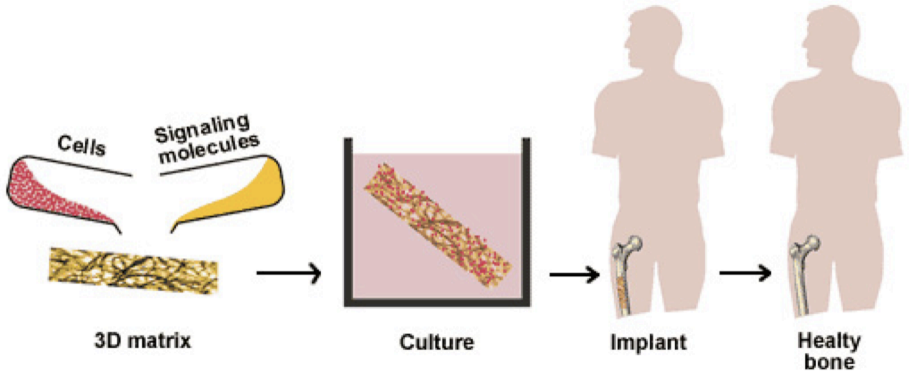

Fig. 8 The three elements together (left of the picture), when in adequate culture, can create a tissue that is suitable for bone reconstruction and, ideally, after implantation, can reproduce healthy bone.

1.4.1 Tissue Engineering for bone reconstruction after tumor resection

Massive bone allografts are widely used in orthopedic reconstructive surgery to replace bone defects due to trauma or oncologic resections. The effectiveness of the clinical outcome depends on bone healing time and type of graft–host integration: the larger the amount of bone to be replaced, the more difficult the integration process becomes (81). This process may involve only 20% of the graft in 5 years, as shown by studies on retrieved allografts (82,

83). In these studies it was shown that revascularization plays a key role to achieve good allograft repair: a delay or absence in revascularization may impair new bone formation and influence the implant outcome. Therefore, accelerating and increasing this process may be critical to allow bone healing and integration.

Numerous techniques have been proposed in order to increase the revascularization, and therefore the regeneration, of a bone allograft. These include the use of stem cells, the use of growth factors (such as BMPs), or the combination of the two.

Bone morphogenetic proteins (BMPs) are a family of pleiotrophic factors, members of the Transforming Growth Factor β super family (TGF-β), that can influence the migration, proliferation, and differentiation of bone progenitors cells (84). It was postulated that the addition of BMPs to an allograft could increase its integration with the host bone by increasing the vascularization. Several studies demonstrated new bone formation when OP-1 alone or in combination with bone marrow is added to the allograft (85-88). BMP-2, BMP-3, and BMP-7 have osteoinductive capacity in vitro and in vivo. In particular, BMP-7, also known as OP-1, has been shown to be able to accelerate bone remodeling, thanks to its osteoinductive and osteoconductive characteristics (89, 90). However, some authors reported that BMPs are able to up- regulate osteoclast-like activity in vitro, thus leading to a higher allograft porosity and resorption when BMPs are added to a massive bone allograft in vivo (55, 85, 91, 92).

Bone marrow contains limited amount of subpopulation of plastic-adherent, fibroblast-like osteoblast progenitor cells, commonly referred as mesenchymal stem cells (MSCs) (93-95). MSCs are used in regenerative medicine to repair bone, due to their ability, demonstrated both in vitro and in vivo, to differentiate into active osteoblasts (96, 97). MSCs isolated from the mononuclear cell fraction after separation by density gradient centrifugation can be expanded ex vivo to reach clinically relevant numbers. It was demonstrated by pre- clinical and clinical studies that MSCs can induce bone repair when associated with natural and synthetic biomaterials (98-104).

1.4.2. Tissue Engineering for soft tissue reconstruction

Plastic surgical techniques continue to evolve to deal with problematic wounds following soft tissue sarcoma resection. Important advances in how tissue is transferred have allowed most wounds to be closed following extirpation; the emphasis is now placed on refining these transfers while minimizing donor site injury. Reconstructive microsurgery has emerged as a frequently preferred way to resurface wounds after sarcoma resection, particularly in patients who have received radiotherapy or previous surgery. Free flaps provide well-vascularized tissue to fill dead space, cover exposed vital structures, and provide structural support and contour. These procedures demonstrate a high success rate of over 90% and often

can ensure a healed wound in a single-stage operation (59, 62, 105). Creative use of the versatile rectus abdominis or latissimus dorsi myocutaneous flaps can reconstruct the majority of extremity, and head and neck soft tissue defects. Endoscopic harvest of muscle flaps has minimized donor morbidity and scarring. The use of "fillet flaps" is an important concept that spares a patient donor site (60, 106, 107). The future holds great promise for sarcoma reconstruction because tissue engineering is rapidly closing in on techniques that can duplicate tissues in the laboratory for ultimate use in reconstruction, thus sparing the donor site from disease (59). In 2007 a group from Singapore has proposed to combine tissue-engineering techniques together with flap prefabrication techniques to generate a prefabricated vascularized soft tissue flap in the nude mice. They have demonstrated that PLGA-c scaffolds, enveloped by a cell sheet composed of fibroblasts, can serve as a suitable scaffold for generation of a soft tissue flap. A ligated arteriovenous pedicle can serve as a vascular carrier for the generation of a tissue engineered vascularized flap (108). In more recent experimental studies, promising new method of generating significant amounts of mature, vascularized, stable, and transferable adipose tissue for permanent autologous soft-tissue replacement have been shown, although this still represents a future prospective (109).

While tissue engineering techniques for soft tissue reconstruction are under study, there is the need for more advanced clinical techniques to improve the quality of the constructs.

2. GOAL OF THIS PROJECTS AND THESIS

The goal of this thesis is to improve the outcome of the current reconstructive therapies for bone and soft tissue pathologies applying tissue engineering strategies. This thesis, therefore, combines two projects that cover:

1. Bone regenerative and reconstructive techniques
2. Soft tissue microsurgical reconstructive strategies

2.1 Bone regenerative and reconstructive techniques

The goal of this project is to improve the outcome of massive bone allograft reconstruction after diaphyseal tumor resection. After the encouraging results of our previous study in the large animal with the use of non-vascularized allograft added with MSC and OP-1, the main goal of this experiment is to obtain a re-vascularization of the

allograft. In our previous study (110) we have demonstrated the superiority of ADSC and OP-1 added to the allograft compared to the allograft alone in the graft regeneration and integration. However, the amount of new bone formation in the allograft remained un-satisfactory. The addition of an artero-venous bundle inside the canal would permit the early re-vascularization of the allograft, allowing the survival of the implanted stem cells and the allograft itself. This, in theory, would end in a graft that can be revitalized and eventually completely replaced by host bone, which will substantially decrease the amount of possible allograft complications. Therefore, the main goal of this project is to accelerate and improve the regeneration of a massive bone allograft through the insertion of a vascular bundle which should guarantee the early and direct re-vascularization of the implant.

2.2 Soft tissue microsurgical reconstructive strategies after tumor resections

The quality of microsurgical plastic techniques for soft tissue reconstruction has improved dramatically over the past years. The goal of this clinical part of thesis is to obtain satisfactory functional, together with aesthetical, reconstruction after resection of main

muscle/tendon for tumor. This will be achieved using motor re-innervated free flaps that should allow the flap to sustain the function of the resected muscle(s) and/or tendon(s).

To our knowledge this is the first study that evaluates the functional outcome of innervated muscle transfer in the setting of irradiated limbs for soft tissue sarcomas.

3. BONE REGENERATION PROJECT

3.1 Background

Bone allografts are widely used to replace bone defects caused by traumas or by surgical operations for congenital malformations, tumor, infections or prosthetic failures (51, 55, 81, 111-115). The incorporation of the allograft requires a cooperative interaction between the allograft itself and the host bone (116, 117). The most important role in the incorporation process is done by the vascular bed which provides not only the cells responsible for the regeneration of this bone (MSCs), but even the fundamental factors for the maturation of these cells and their differentiation into osteoblast precursors (48, 85, 86, 101, 118, 119). The allograft incorporation can therefore be seen as a process that involves both re-vascularization and re-vitalization at the same time, because these two actions are strictly connected one to each other. A slow or insufficient re-vascularization can impair the implant incorporation or, in some cases, cause its failure. On the other hand, when a good re-

vascularization of the graft is achieved, the initial integration of the implant is shown by a sound consolidation at the level of the osteotomy lines, which, in turn, will lead to a satisfactory incorporation of the implant and the decrease of the risk of failure.

It is clear, therefore, how vessel penetration into the graft constitutes the primary and probably most important process in the allograft incorporation. The re-vascularization can be achieved mainly in two ways: 1) the effect of the surrounding tissues and structures, which stimulate the vessel penetration with an extrinsic mechanism (120-122); or 2) by implanting a vascular pedicle inside the graft, which directly brings the vascular supply together with all the nutrient and growth factors (123). This second mechanism can guarantee a more direct and secure method to re-vascularize the graft, while the extrinsic mechanism tends to be a slow and incomplete process with a limited power. Together with blood and growth factors supply, there is the necessity to introduce progenitor cells, which can in turn act as stimulators for other cells and growth factors and, in a second time, differentiate themselves into bone forming cells (101).

The presence of a high number of Stem Cells into the area that need to be re-vascularized and re-generated is another key factor in the integration process: these cells, in fact, primarily act as cytokine producing elements which are responsible for the vascular neo-formation. The Vascular Endothelial Growth Factor (VEGF), for example, is one of the main cytokine produced by the MSCs, and acts

not only as a neo-angiogenic factor, but even as an osteogenic factor. Other factors produced by the MSC, which have an autocrine effect as well, are TGF-β, which act as important osteogenic factors (73, 124).

Stem cells are relatively easy cells to obtain in patients that undergo to orthopaedic surgery procedures, as they can be obtained not only from a bone marrow aspirate, but even from the sub-cutaneous adipose tissue (75). Moreover, these cells are safe and they do not constitute a risk in terms of rejection because they can be used as autogenous cells.

BMPs are members of the TGF-β superfamily which has been demonstrated to play a fundamental role in skeletal formation both in embryonal growth and in adult life (125, 126). This is true especially for BMP-2, BMP-4 and BMP-7, even if only BMP-2 and BMP-7 (also known as OP-1) have demonstrated their osteo-inductive potentiality in pre-clinical and clinical experimental studies (91, 119, 127-130).

In a bone regeneration setting such as delayed fracture, aseptic bone necrosis or other critical defect, bone morphogenetic proteins (BMPs) have proved key in enhancing the natural ability of the surrounding tissues to produce bone healing (84, 91, 129, 130). If the mechanical conditions are fulfilled, these molecules are able to address progenitor cells in the bone-forming cascade to allow the repair of the damaged tissue. It is important to underline also the action of single BMPs as well as in association with others of the BMP family group. It has been stated that BMPs produce bone by a complex

series of events involving a subset of proteins all capable of inducing bone formation by themselves, including BMPs -2, -3, -4 and -6. Concurrently other cytokines that are not BMPs may facilitate bone formation in other ways, e.g. FGF, which has an angiogenic effect that promotes neovascularisation, and PDGF and IGF-1 acting as local modulators (131).

3.2 Previous studies

It is clear since many years that the main reason of allograft failure is its poor blood supply; therefore, because early vascular invasion is a key factor in bone allograft incorporation, in our very first study done in 2005 we tested whether the combination of MSC and platelet-rich plasma (PRP) was able to increase vascular invasion and massive allograft integration in a large animal model (101). In this study we have chosen PRP because on activation, platelets release a high concentration of a variety of proteins including VEGF and fibroblast growth factor-2 (FGF-2) (132, 133). These molecules released by platelets are strong angiogenic inducers and are known to be mitogenic for the MSC (134). In this early study, we were able to demonstrate that MSC contained in a collagen and PRP-based scaffold can improve allograft integration, even though this improvement was moderate (Fig. 9). Being both MSC and PRP angiogenic factors, we

moved to a second study in which we wanted to couple an osteoclastic activity to an osteoblastic one, still maintaining the strong angiogenic stimulation.

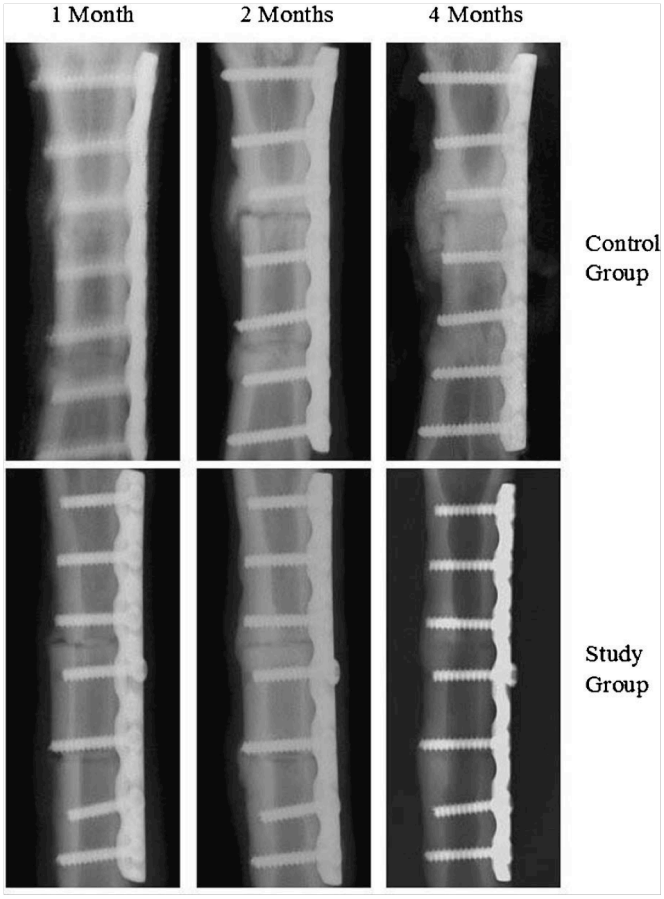

Fig. 9 The evolution of the healing process in the control and study (MSC + PRP) groups is shown in radiographic images. At both 2 and 4 months there is less evidence of healing along with more periosteal callus in the proximal osteotomy line in the control group, while a more direct healing process is seen in the study group .

In our subsequent study, therefore, using the same large animal model (sheep), we tested whether MSCs and OP-1 added to a massive bone allograft can promote a complete bone–allograft integration (110). The idea behind the project was that the regeneration process should be achieved by direct penetration of cutting cones at the junction site from the host bone to the allograft stimulated by OP-1, together with the strong angiogenic activity promoted by this protein, thus reducing the amount of callus formation around the implant. In the meanwhile allograft bone should be completely replaced by newly formed bone promoted by the MSCs activity.

In this study we evaluated the effect of OP-1 alone (BMP-7), MSCs alone and OP-1+MSCs in the allograft integration of a massive intercalary defect in the sheep's metatarsal bone. Interestingly, we noticed that when OP-1 was used alone, the amount of bone resorption was substantially higher than the ability of the host bone to stimulate new bone formation, thus leading to an almost complete disappearance of the allograft in some cases. This high absorption activity can be explained by the strong stimulation of the osteoclastic activity by the OP-1, without a sufficiently high coupling with osteoblastic activity. On the other hand, when MSCs where added to OP-1, the osteoblastic function was strong enough to produce a very good amount of new bone in the area previously re-absorbed by the osteoclasts (Fig 10). Finally, when MSCs where used alone, the amount of new bone formation was minimal (but still higher compared to the control group with no OP-1 nor MSCs) due mainly to the limited

activity of these cells in a contest where there is no absorption and, more importantly, where there is insufficient vascularization to allow them to survive enough time to complete their tasks.

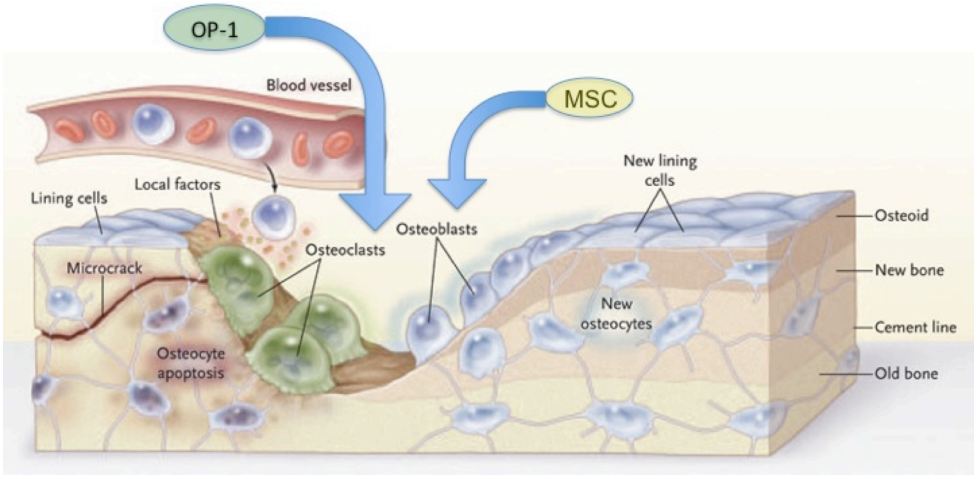

Fig. 10 Schematic effect of OP-1 and MSC on bone. While OP-1 stimulates osteoclastic activity and, thus, bone resorption, MSCs, by their direct differentiation into osteoblasts or by direct stimulation of circulating cells to recruit osteoblasts, stimulate bone formation.

This results were consistent with the findings of Cullinane et al. (85) who demonstrated a significant reabsorption rate with a massive bone allograft in a canine model treated with rh-OP-1 at 12 weeks postoperatively. The results obtained in this further study were very similar to those of their previous study, confirming the high stimulation of graft reabsorption when rh-BMP-7 was used (86). This

concept was also confirmed by other authors dealing with experimental model of impaction grafting using morcellised allograft and BMP (135).

In our previous study (110) we were able to demonstrate that the addition of MSCs together with OP-1 is able to increase the integration of a massive bone allograft with a high amount of new bone formation inside its structure (FIG 11)..

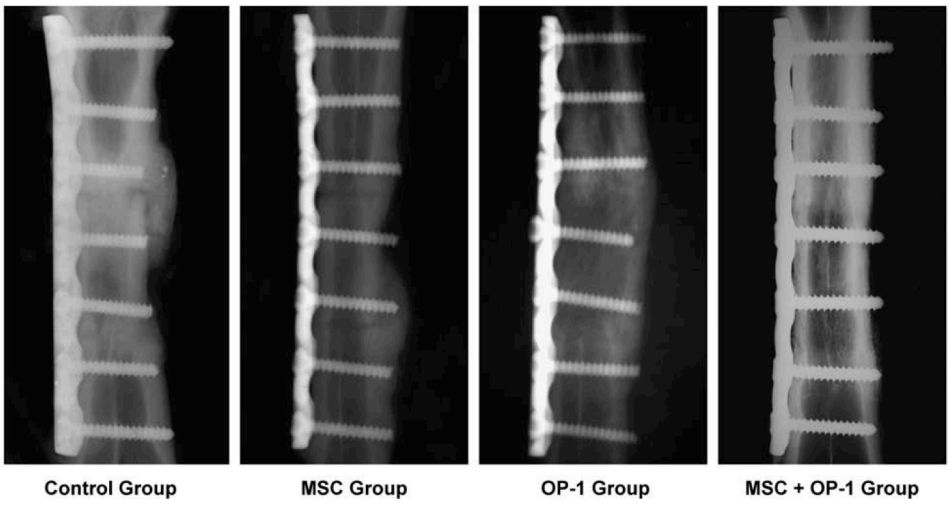

Fig. 11 X-Ray evaluation of groups treated with MSC alone, OP-1 alone, and the combination of MSC and OP-1 at 4 months. When MSCs were used alone, although a better bone integration was seen compared to the control group, the osteotomy lines were still visible and the integration occurred via external callus bridging. When OP-1 was used alone, a great amount of bone allograft resorption was evident. On the other hand, the combination of MSC and OP-1 allowed a far better integration with a direct process and restoration of the medullary canal and cortex continuity.

The role of the timed coupling between bone reabsorption (osteoclasts) and bone formation (osteoblasts) is of paramount importance in bone tissue engineering. However, despite the outstanding improvement compared to the actual allograft incorporation strategies, this technique was still not powerful enough to obtain a complete allograft revascularization and regeneration. We believe that the main obstacle for a complete regeneration process is the lack of sufficient vascularization, which in turn decrease the chance of survival of the implanted stem cells

The activity of OP-1 and MSC to promote early vascularization was noted not only on Xray, but even on histology, and was encouraging (Fig 12). However, this re-vascularization was noted to be still insufficient, and this probably was due to the extrinsic mechanism by which the new vessel formation has to develop.

The idea, therefore, was to implement the vascularization of the graft with a direct mechanism, by implanting an artero-venous pedicle within its structure, in order to achieve an early blood supply that can 1) support the function and the survival of stem cells, and 2) implement the supply of circulating cells, such as macrophages, to increase the bone remodelling thanks to their paracrine effects. For this purpose we developed a small animal model that is the focus of this first part of this thesis.

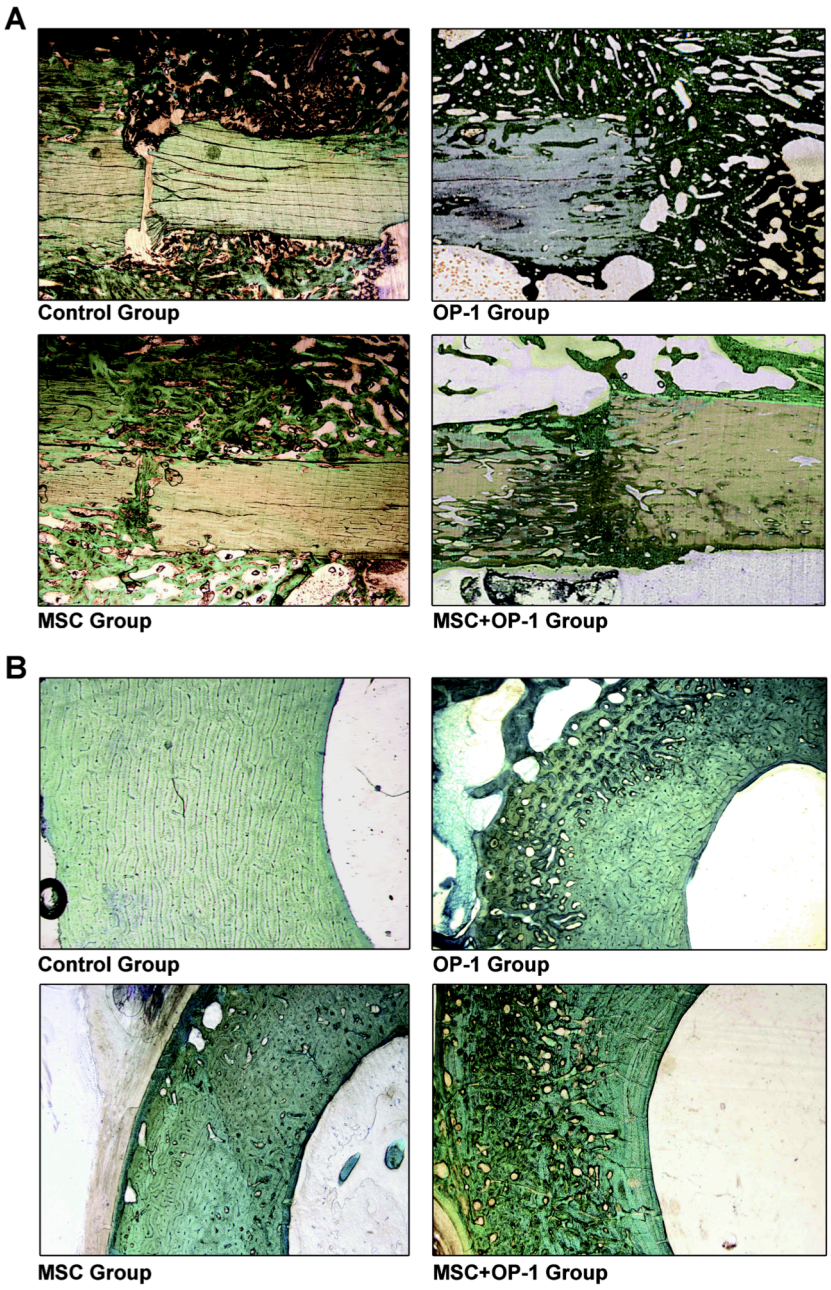

Fig. 12 Histology images of the frontal and axial cuts in the four groups (Trichromic Masson; optical zoom: 1.25×). Only the cortex opposite to the plate side is included in the slices. In the control group abundant periosteal callus was evident with lack of newly formed bone inside the allograft cortex. In the MSC group there was more rearrangement inside the allograft cortex with no evidence of cutting cones, and marked periosteal callus. In the OP-1 group the allograft was completely absorbed with a marked presence of woven bone and bone remodeling. In the MSC + OP-1 group the bone remodeling inside the allograft was predominant with several cutting cones starting from the host cortex invading the grafts cortex. The callus was almost not visible.

In a study conducted very recently by a German group (123) an inguinal artero-venous bundle was dissected in the groin of female white New Zealand rabbits (n = 6) and placed centrally inside a scaffold seeded with Adipose Derived Stem Cells (ADSC) via a central drill hole. In the same surgical session this construct was placed into a segment of a-vital cortical bone allograft from a donor rabbit. Un-seeded scaffolds that were implanted and treated in the same fashion served as controls (n = 6). In order to prevent external revascularization, all constructs were wrapped in silicon foil and finally implanted in the rabbits' groin. Three months later, the constructs were explanted and investigated for vascularization of (a) the scaffold (b) the surrounding bone allograft. As a result, ADSC seeded scaffolds showed a significant increase in new vessel formation in the scaffold as well as in the bone allograft compared to un-seeded scaffolds. Furthermore, new vital osteocytes as a sign of cellular repopulation inside the bone allograft were found only in the treatment group. Vital chondrocytes were only found in the ADSC seeded scaffolds as well. This study was very encouraging for our project, as it demonstrated that the introduction of an artero-venous bundle inside the centre of a scaffold improves its revitalization; in our approach, we aimed to regenerate new bone directly into the defect, as it would be more reasonable in a clinical situation. After the design of the model, the introduction of stem cells coupled with Bone Morphogenetic Proteins, specifically OP-1, should considerably implement the revitalization of a massive cortical allograft.

3.3 Experimental design

Rabbits were considered as the ideal starting point for our experimental project. These animals are, in fact, very easy to handle, gentle in their temperament and relatively quite in the post-operative period, with a relatively good pain threshold. At the same time, they are economic to use and this characteristic allow the use of a sufficient number of animals to achieve statistically sound results in a relatively short amount of time. The most important limitation of this animal is however the poor transferability to the human clinical situation due to the anatomical differences not only as a general concept, but even in the bony anatomy, bone strength modulus and cortex/medullary canal ratio in the diaphisis. Together with this, even the bone regeneration physiology is different, and this represents another difficulty in the comparability with humans.

It will be therefore necessary to add a second phase to this preliminary project, which will involve the use of larger animal models that can guarantee a better transferability of the results to humans. This model will be the sheep metatarsal one, which has been proven to be extremely reliable and repeatable in our hands.

In this preliminary project we used 16 rabbits; there was the need to use 4 of these animals to test the model, for two main reasons:

1) it was the first time, in our hand, that we created an intercalary massive defect in the femur of the rabbit, and 2) in all the previous publications where a femoral diaphyseal defect was created, the fixation method chosen was an intra-medullary pin. In our case we could not use this fixation device because the main goal of our project is to stimulate the re-vascularization by putting a vascular pedicle inside the medullary canal of the femur, therefore a different fixation method had to be chosen. The first 6 animals, therefore, were used to test the surgical model; the remaining 10 were divided into 2 groups of 5 each. In the Group A the allograft was used alone and this represented the control group; in Group B, the artero-venous bundle was inserted in the femoral canal of the allograft. In this preliminary study, no cells or growth factors were used; once it is demonstrated that the vascular pedicle insertion improves the revascularization of the allograft, the groups with MSCs and OP-1 will be performed. The chosen experimental time was 6 weeks as an early time point. Being the goal to obtain early regeneration and re-vascularization, 6 weeks has been identified as the best time to evaluate the results. Earlier and later stages will have to be considered in the future.

3.4 Materials and Methods

a) Surgical Procedure

The surgical procedure is performed with the animal under general anaesthesia and antibiotics coverage. After shaving the limb and the groin area, the animal is placed supine and a longitudinal incision at the groin is performed in line with the inguinal canal. The femoral neurovascular bundle is exposed and isolated, and, following the femoral artery proximally, the superficial epigastric vessels are identified and isolated. These vessels are followed up to their proximal end through the groin fat, and, maintaining their perivasal tissue, are ligated after heparinization at the most proximal end using a nylon 4/0, trying to maintain the highest possible length of this pedicle (Fig 13). The suture stitch, at the same time, is kept long at least 10 cm, in order to allow in the second time the correct positioning of the pedicle in the femoral medullary canal. The isolated pedicle is temporarily rested in the wound and protected with moist gauze.

A second incision is performed as a lateral approach to the femur. The fascia is sectioned and via smooth dissection the diaphysis of the femur is approached passing through the vastus lateralis and the semimembranosus muscle. Using a high speed saw, a critical defect (136) of 1,5 cm is created in the mid-shaft of the bone, taking care not to create any micro-fracture in the very fragile cortex.

The defect that is created is thereafter filled with a bone allograft of the same dimensions previously harvested from another animal and stored in sterile conditions at -80 degrees.

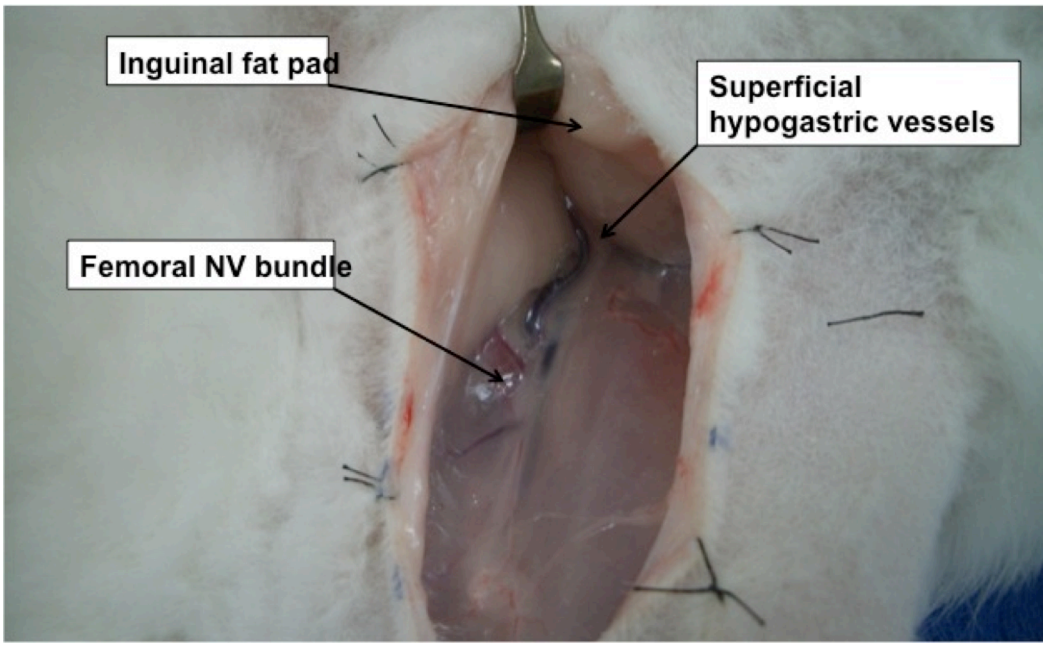

Fig. 13 Anatomy view of the superficial hypogastric vessels in the rabbit. Approach to the antero-medial compartment of the thigh, the femoral neuro-vascular bundle is identified and the superficial hypogastric bundle is showed just underneath the inguinal fat pad.

Before fixing the allograft, a 0.8 drill is used to create a hole in the anterior cortex of the host bone, just proximal to the osteotomy line; the vascular pedicle previously harvested is heparinised again and then passed through this hole. A second hole is made in the distal part of the host bone, just distal to the second osteotomy line. The

vascular pedicle is therefore inserted inside the allograft canal and, using the long suture previously maintained, it is passed through the distal hole and then fixed at the level of the distal diaphysis. The allograft is then fixed using plate and screws on the host bone, and using cerclages on the allograft bone in order not to weaken it.

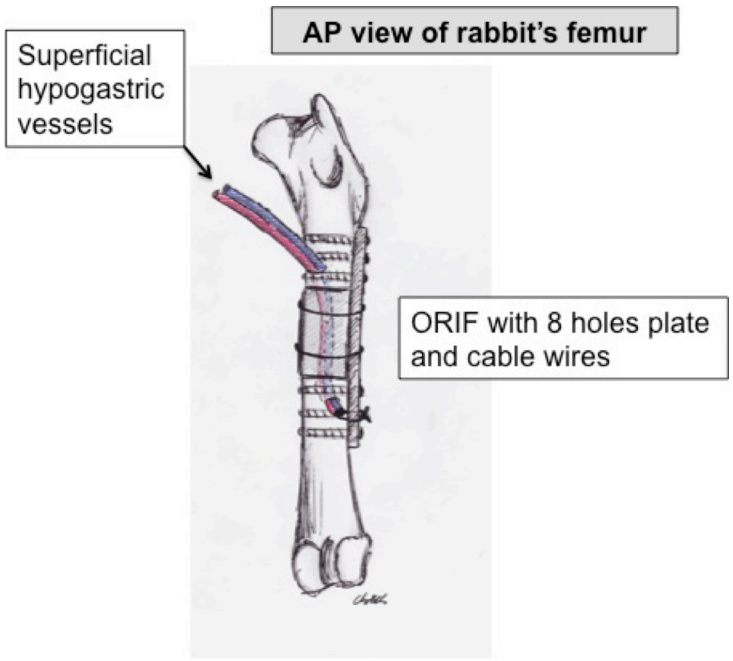

Fig. 14 Schematic picture of the allograft position and fixation. The defect is created in the mid-shaft of the rabbit's femur, the allograft is fixed with plate, screws and cable wires. The artero-venous bundle is inserted through a proximal hole into the medullary canal, and fixed at its exit from the distal hole in the cortex.

In a second time, once the model will be confirmed as reliable and repeatable, the injection of MSCs and OP-1 can be done using one of the screw holes, using the procedure already used in our previous study (101). Once a sound fixation has been achieved, the wounds are closed and the X-Ray check is performed.

One limit in the use of the femoral model in the rabbit is that it doesn't allow a post-operative cast protection, which instead is possible in the metatarsal model in the sheep. The cast provides stability and protects from rotational and compression stresses which may produce an early failure of the fixation. In the rabbits, being this protection impossible to obtain, the risk of early failure is therefore increased and needs to be taken in consideration. Together with this, the extreme friability of the cortical bone of the rabbit and the very thin cortex compared to the medullary canal contribute to the high risk of fixation instability.

b) Analysis of results

The goal of this preliminary project is to demonstrate an improvement in bone allograft integration through an improvement of the neo-vascularization of the graft. The analysis performed therefore was focused on the appearance of the bone, the histology and the viability and patency of the vascular pedicle.

We aimed to evaluate the appearance of the implant using Xray, the viability and patency of the transposed vascular bundle, and the amount of new bone, its integration with the host, and the patency of the implanted vessels using histology and histomorphometry.

1. X-Ray: In order to investigate the allograft integration all the animals were radiographed in AP view projection with a portable X-ray machine (Franceschini, Italy) with the following program: 70kV, 60mA, 0.25s. Hence, the images were digitised to achieve a better definition and evaluation. As in our previous works (101, 110, 119), we designed a simple score ranging from 0 to 2 in two different categories: osteotomy healing and periosteal callus presence. In the first category 0 was assigned when there was discontinuity in the osteotomy line, 1 for continuity but the line was still present, and 2 when the line was hardly recognizable. In the second category concerning periosteal callus, 0 equal to no evidence of periosteal callus, 1 to moderate presence, 2 to marked and excessive presence.
2. Macroscopically appearance of the vascular bundle: At the time of harvest, the patency of the vascular bundle was analysed: with the animal in anaesthesia the pedicle was first identified at the proximal entry point

and the pulse was recorded, then the artery was sectioned to establish blood flow trough it lumen.

3. Histology and histomorphometry: The whole femur was explanted and, once freed from the soft tissue and once the plate was removed, fixed in Bouin and then in 4% paraformaldehyde before being cut (Exact Parallel System, Hamburg, Germany). As reported before (101) the osteotomy lines were sectioned in a frontal plane passing through the polar line including part of the grafted bone. The remaining central part of the allograft was cut in a transverse plane (Fig 15). All the specimens were embedded in methylmethacrylate (MMA) to achieve precision cuts and further grinding. The result was a 40–70 micron thickness slides. Staining was achieved with trichromic Masson technique (toluidin blu, acid fucsin, fast green). The slides were evaluated with the Qwin-Leica Imaging System LTD (Cambridge, England) and digitized for histomorphometrical analysis.

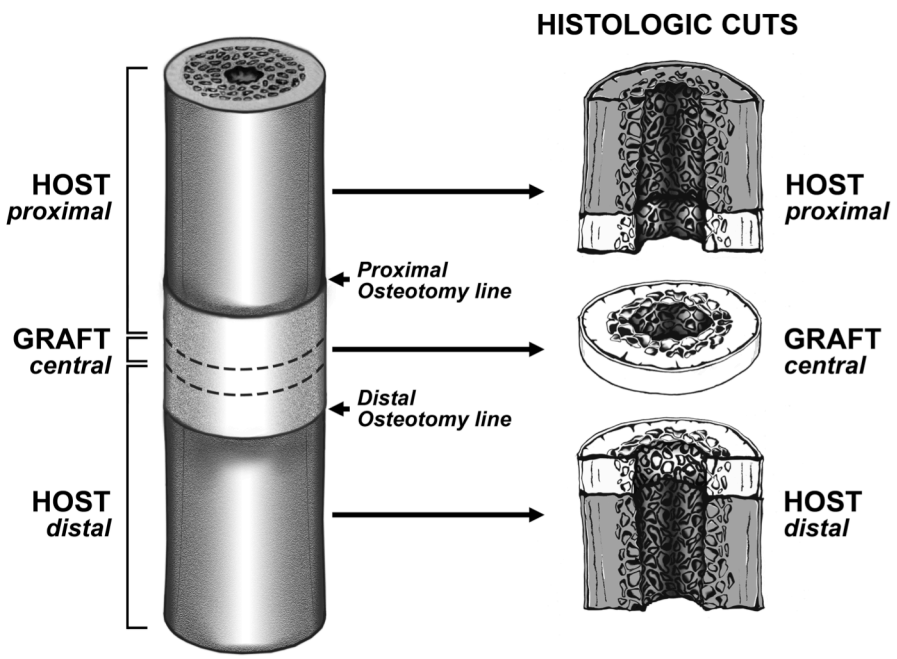

Fig. 15 Schematic picture of the histology cuts performed. The most central part of the allograft, with the vascular pedicle inside it, is examined using axial cuts. The proximal and distal osteotomy lines are examined through a sagittal cut which includes the passage between the host bone and the allograft.

The following parameters were evaluated: (1) percentage of new bone in the allograft, (2) percentage of callus surrounding the osteotomy and (3) penetration of vessel in the graft from the osteotomy line. Together with this, the patency of the implanted vessels was evaluated using Hematossilin-Eosin staining in paraffine embedded section of the vessels at the level of the mid-shaft of the allograft.

3.5 Results

a) Animal model

Despite rabbits are simple animals to work with, the femoral intercalary defect presented several challenges that made the repeatability of the model a difficult goal. All the fixation techniques used showed difficulty on the achievement of fixation stability, mainly due to the brittle characteristics of the rabbit bone.

- Plate and screws, no cables. 2 animals

The first attempt was done following the protocol of the well established metatarsal model in the sheep. A 8 holes LCP plate was chosen and fixed to the bone with bi-cortical screws, however the fragility of the bone in both the allograft and the host site did not allow a stable and sound fixation. The bone, in fact, tended to crack with the drill and, even more, with the compression of the screws, despite a good position of them. In both the animals a fracture in the proximal and distal femur was evident at day 1 post op, therefore the animals had to be sacrificed.

- Plate, screws and metallic cables. 4 animals

The following 4 animals received fixation with a 6 holes LCP plate, 2.7 mm (small fragment) screws (2 in the proximal host bone and 2 in the distal) associated with 0.8 Charnely wires used around the plate and the allograft as further stabilizer. This choice was made in order to avoid excessive drilling of the allograft, therefore avoiding possible fracture propagation. With this method, a better fixation was achieved.

Despite the good post-operative result, loss of fixation was noted at the allograft-host junction respectively after 5, 7, 14 and 17 days post-op, suggesting that the metallic cables did not confer enough stability to the construct and that the fixation at the level f the host bone was not achieved with the use of only 2 screws (Fig 16).

The difficulty in the use of the metallic wires was noted especially in their tightening, as an excessive force would have resulted in the fracture of the graft or the host cortical bone. This, in fact, happened in one animal. On the contrary, when the wire was left not excessively tightened, the loss of fixation was the result after few days. The fact that the animal was free to weight bear in the immediate post-operative period led to excessive rotational stresses at the femur, with increased chances of early failure.

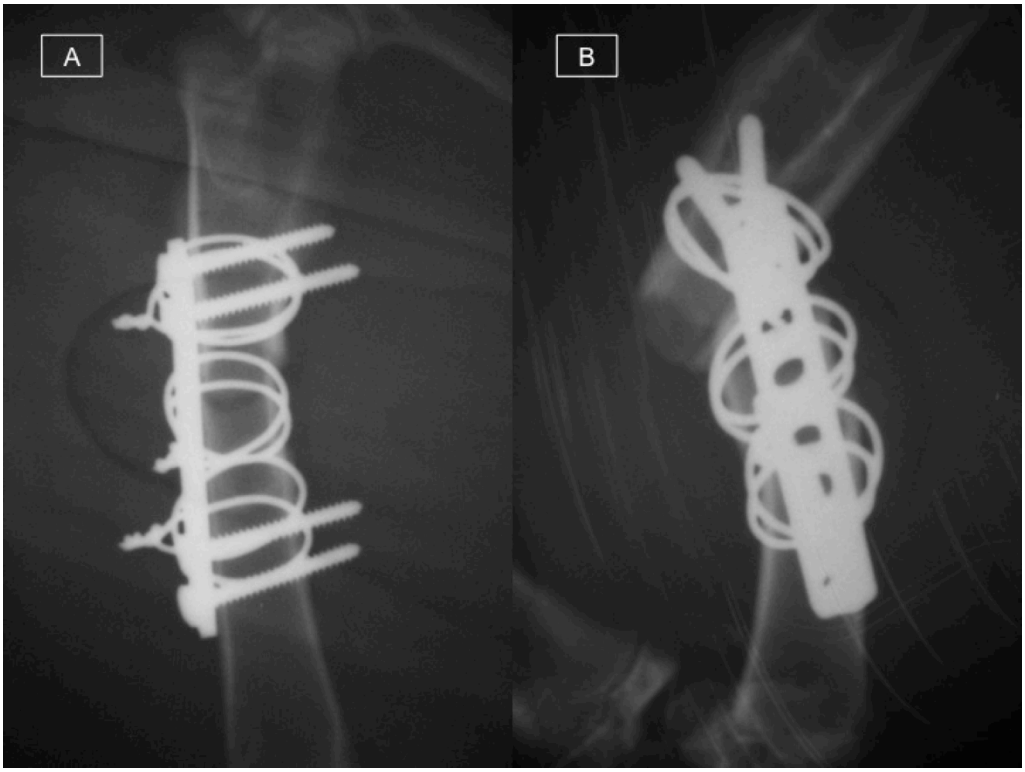

Fig. 16 Xray of the femoral allograft fixed with 6 holes DCP plate, screws and metallic cable wires. A) Post operative check, with satisfactory position of the allograft and apparent satisfactory fixation. B) 1 week post-operative Xray check, loss of fixation at the proximal osteotomy.

- Plate, screws and plastic cables. 10 animals

After the failure with metallic wires, we improved our fixation using plastic self-locking cables associated with a longer plate (8 holes). The cables allowed a good primary stability and decreased the risk of over-tightening and iatrogenic fracture. The difficulty

encountered in the use of these cables was their sterilization: being in fact made of standard plastic, high temperature sterilization process was not possible, and they were sterilized via soaking into betadine and alcohol for 30 min. The longer plate and the fixation with 3 screws both in the proximal host bone and in the distal allowed a better stability as well. All the following animals were treated with this technique (5 group A, 5 group B) (fig 17).

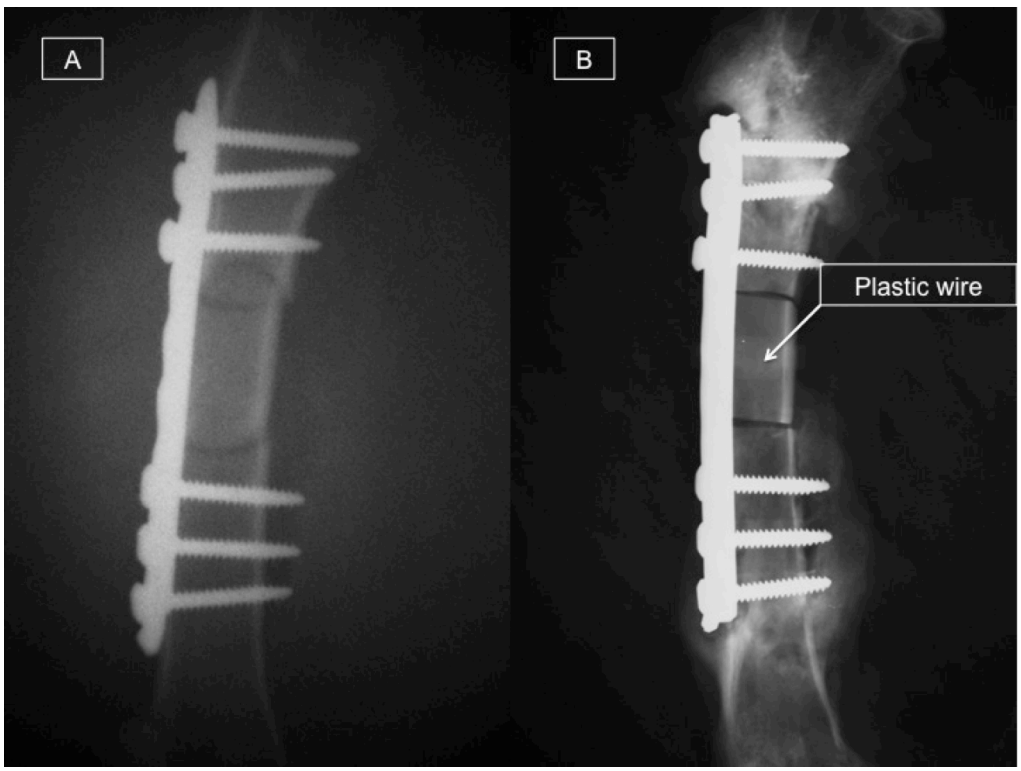

Fig. 17 Xray of the femoral allograft fixed with 8 holes DCP plate, screws and plastic cable wires. A) Post operative check, with satisfactory position of the allograft and apparent satisfactory fixation. Note the 3 screws (6 cortices) fixation both proximal and distal in the host bone. B) 6 week post-operative Xray (femur harvested at the end of the experimental time), good fixation maintained at the proximal and distal osteotomies. Note in transparency the shape of the plastic cable. Note the big amount of callus around the screws and the lack of healing at the osteotomy sites.

b) Clinical Complications

Despite a good and satisfactory intra-operative allograft fixation in the animals treated with 8 holes plate, screws and plastic cables, some complication still occurred. There were 2 infections (1 in Group A and 1 in Group B), and 3 late fractures (2 in Group A, 1 in group B). These animals had to be sacrificed before the end of the experimental time and therefore were not evaluated. As a consequence, these complications led to a small number of animals available for analysis.

The remaining animals available for evaluation were: 2 group A and 3 group B. Because of the poor clinical outcome and the difficulty on the repeatability of the surgical procedure, for ethical reasons the model had to be abandoned and no further attempts were performed, with a following plan of moving to a larger animal model (sheep) already well consolidated.

c) Macroscopic evaluation

When no complications occurred during the 6 weeks of the experimental time, the femur was dissected free form the surrounding soft tissue, disarticulated at the hip and knee levels, and harvested for analysis. At the time of harvest, despite the good position of the allograft and the absence of clinical infection, in all animals regardless of the group, an important amount of macroscopically reactive tissue was evident in the area of the plastic cables. An abundant and

hypertrophic callus was present at the level of the screws, both proximal and distal, while no callus was noted at the osteotomy lines. The allograft showed macroscopic evidence of being constituted by sclerotic bone with absence of remodelling around the osteotomy lines (Fig 18).

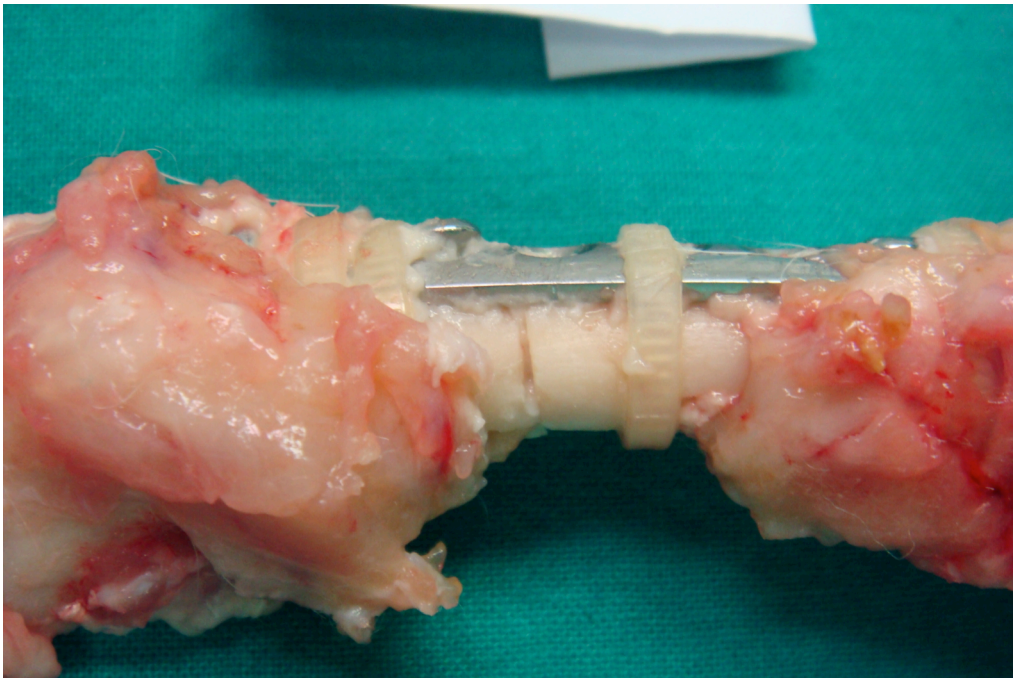

Fig. 18 Macroscopic appearance of the femur after its harvest. The allograft is maintained in acceptable position by the plastic cables, but no integration is seen at the osteotomy lines. Big callus/soft tissue formation is noted at the proximal ond distal host bone.

d) Transplanted vessel evaluation

At the time of harvest, before the whole femur was retrieved, the patency of the vascular bundle was analysed: with the animal in anaesthesia the pedicle was first identified at the proximal entry point and the pulse was recorded, then the artery was sectioned to establish blood flow trough its lumen.

In 2 of the cases belonging to Group B the pedicle was shown to be not pulsatile, and after the vessels section no blood flow has been shown. The lumen was thrombotic at the proximal level of the pedicle starting from the emergency of the branch from the femoral vessels. No clear torsional movement at the level of the superficial hypogastric vessels emergency was evident, but the thrombotic process was noted starting at the emergency of the branch. It was clear, therefore, that the allograft did not receive any blood supply. In one case, however, the vascular bundle showed to be patent with good flow after its section.

Despite the vessels occlusion in 2 out of 3 cases, the subsequent imaging examinations were still performed because no clear timing on the thrombotic process was known, and therefore some early vascularization could have still happened.

d) Imaging

The rabbits were evaluated radiographically and histologically. The small number of sample did not allow any statistical analysis. On X-Ray exam, no difference was evident between the two groups of animals with regards of callus formation or bone/allograft integration. In both groups, an abundant callus was noted starting from the proximal host bone, just proximal to the first screw, which indicates an attempt of fixation via external bone bridging (Fig: RIV 15 and 16.. will be group A and B).

	6 weeks			
	prox		***dist***	
	Callus	*Healing*	*Callus*	*Healing*
Group A (Control)	1	0	0	1
	0	0	1	0
Group B (Pedicle)	1	0	0	0
	1	1	2	1
	2	0	1	0

Legend:

CALLUS	**HEALING**
0: Low	0: No continuity. Visible and separated osteotomy lines
1: Medium	1: Callus continous. Osteotomy lines visible
2: Hypertrofic	2: Healing with cortical and midollar continuity

Fig. 19 Xray results for control and study groups. There was no relevant difference between the two groups in terms of callus formation nor healing at the osteotomy lines.

e) Histology

The histology has been performed in the available animals. The amount of bone formation in the allograft and the quality of consolidation has been studied.

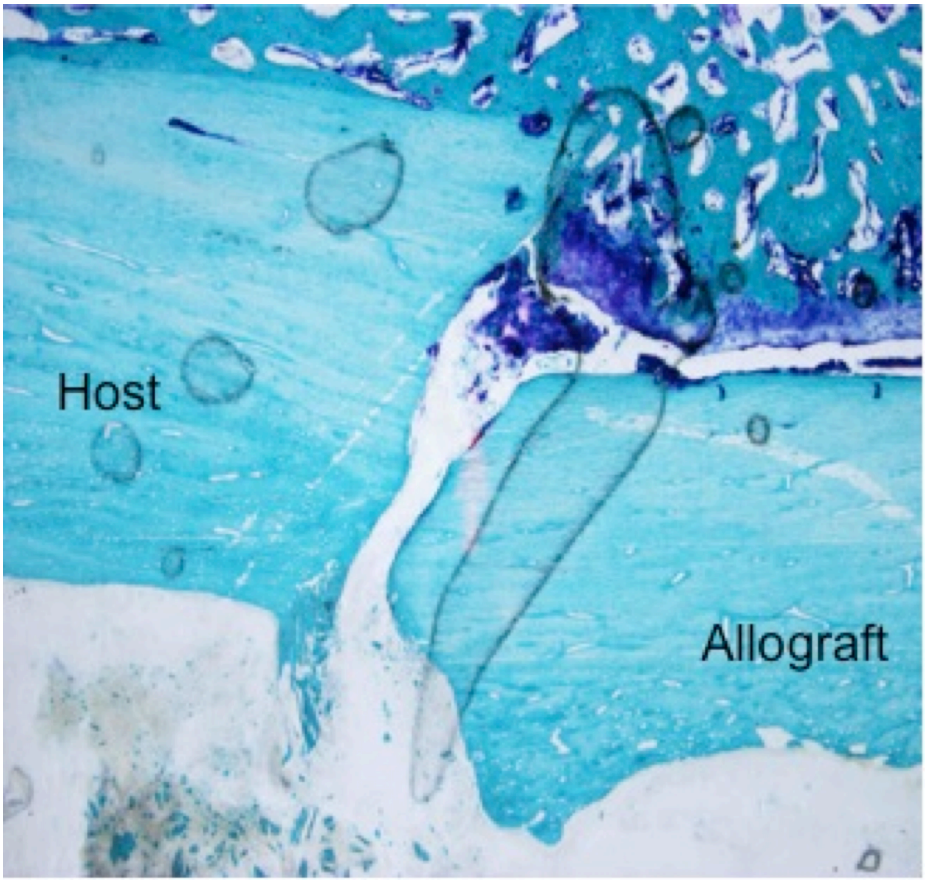

Fig. 20 Histology of a longitudinal cut at the level of the osteotomy line in Gourp A (Control). Abundant callus is noted. The gap between the host and the graft bone is still present with no evidence of allograft remodelling. 1,25 x, Metacrilate embedding, Masson Trichromic staining.

In group A the histology showed an abundant callus formation around the proximal and the distal osteotomy line. The callus was formed by woven immature bone with a component of fibrous tissue. The allograft in all cases showed no new bone formation and did not show signs of remodelling; at the level of the osteotomy sites, a gap between the host and the graft was present with minor fibrous tissue. (Fig 20) There was no evidence of revascularization process.

In Group B the patency of the artero-venous pedicle has been shown to be deficient in 2 out of 3 animals. In those 2 animals the histology picture was comparable to the ones seen in the control group, with no evidence of allograft remodelling. In the animal were the pedicle was patent, there was evidence of new bone formation in the medullary canal with some endosteal bone remodelling (Fig 21), and this was evident both at the level of the osteotomy lines and at the more central part of the allograft (Fig 23).

With regards to the vascular pedicle, in the cases were it was macroscopically not pulsatile the histology showed an occluded artery with a full thickness thrombus. The vein, on the other hand, showed patency but no evidence of blood in its lumen. In the only case in which the pedicle showed to be pulsatile, histology confirmed the patency of the artery with no thrombotic process.

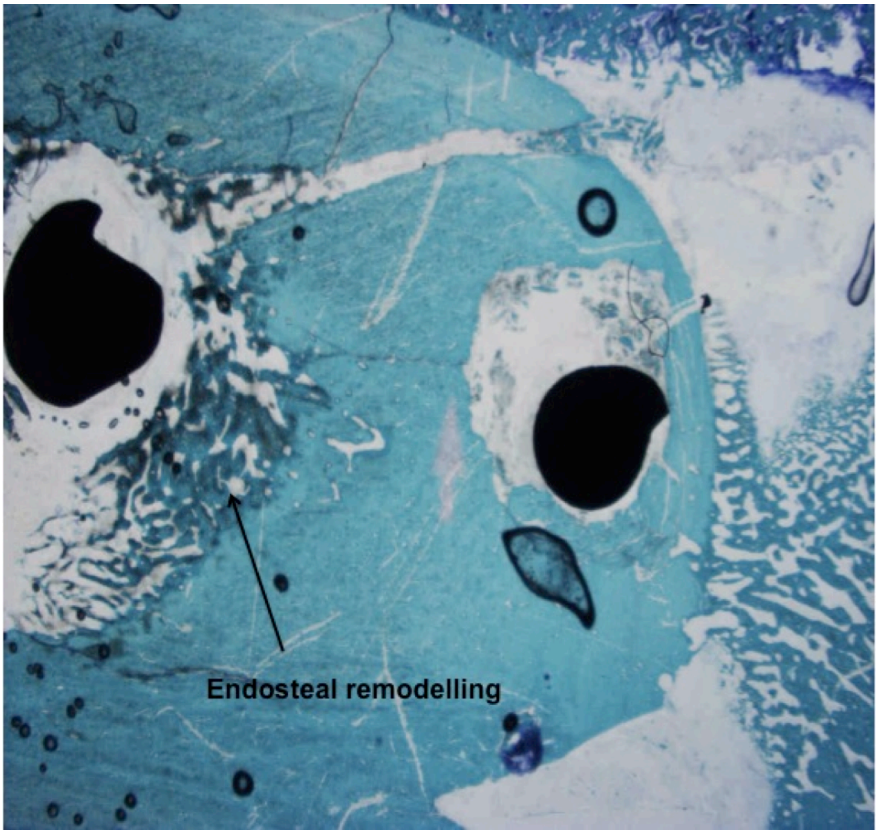

Fig. 21 Histology of a transverse cut in the allograft in Group B with patent vascular bundle. Endosteal bone remodelling is noted with new bone formation and new vessel formation. 1,25 x, Metacrilate embedding, Masson Trichromic staining.

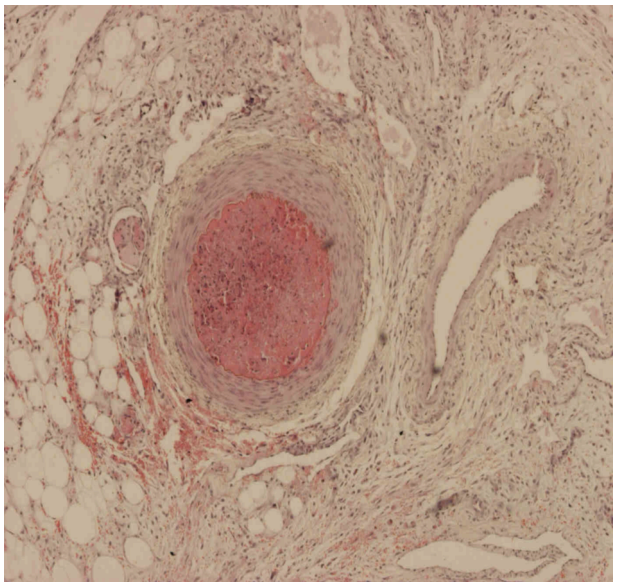

Fig. 22 Histology of vessels; the artery spears occluded by a full thickness thrombus, while the vein is patent. Magnification 2 x, Paraffine embedding, H&E staining.

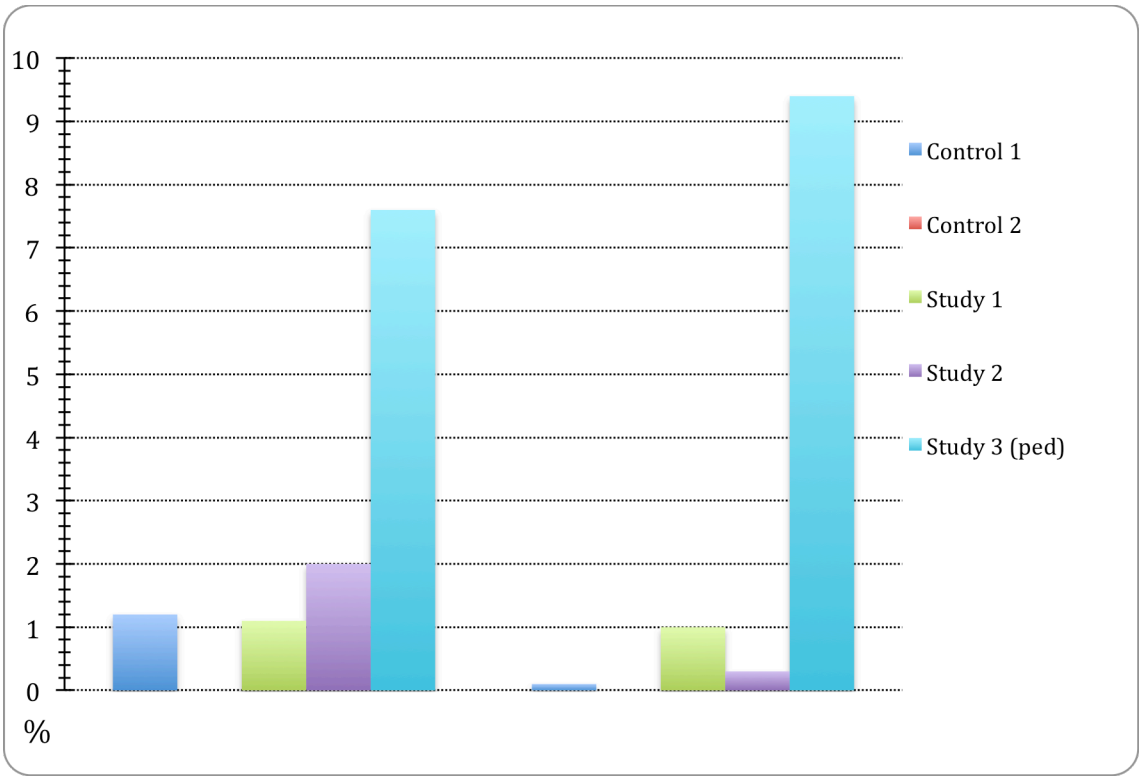

Fig. 23 Table representing the amount of new bone at the level of the osteotomy lines in the longitudinal cuts, and at the level of the central part of the graft in the axial cuts. The values are expressed as a percentage of the total allograft bone in the histology slice. In the animal with the patent pedicle (Study 3) there was substantial more new bone in both areas compared to all the other samples.

3.6 Discussion

The concept of scaffold re-vascularization to promote its integration into a tissue and its revitalization has been known since many years. Vessel re-implantation into avascular bone is already a very well known clinical strategy in the treatment of bone osteonecrosis such as Kienbock disease (137) or scaphoid avascular necrosis (138, 139). More recently, some studies have been focusing their attention on the possibility to introduce a vascular pedicle into a bone allograft to stimulate its remodelling and, by adding stem cells and/or growth factors, to further improve the new bone formation (120, 123, 140). These studies showed that the implantation of a vascular pedicle into a stem cells seeded scaffold noticeably improves neo-vascularization and osteocytic repopulation of previously a-vital bone allograft. These results have been obtained in a not loaded condition, such as the subcutaneous pouch of the animal. The outcomes of these studies are very encouraging, and therefore the application of this technique for the revascularization of the allograft in a loaded condition would add substantial benefit. The main reason for this is the possibility of immediate clinical application, while obtaining a remodelled bone in a subcutaneous pouch remains ethically controversial and clinically more difficult to perform.

A good animal model is of paramount importance to obtain reliable results in an in-vivo experimental protocol. Following our

experience with the sheep, the rabbit femoral model seemed to be a very good small animal model to start our project. The concept of our reconstruction did not allow us to perform the fixation method that is most common in literature (141, 142), which involves the use of an intramedullary pin. This has been considered a reliable and reproducible technique, however the insertion of an artero-venous pedicle inside the femoral medullary canal made this procedure not possible. Therefore, we had to use the plate and screws internal fixation technique, which is the same that we used in the large animal model and is the same that would be used in humans. Unfortunately, the characteristics of the rabbit bone, its friability and the reduced ratio of cortex thickness/medullary canal diameter at the level of the femoral diaphysis made the procedure technically very challenging. Together with the difficulty of the procedure, the high rate of intra-operative and post-operative complications has been shown. A consideration that has to be made in this contest is the impossibility to obtain a post-operative protection bandage or cast in the rabbit femur, which evolves in the animal free to move after surgery. In the sheep, in fact, when an intercalary defect is performed in the metatarsal bone, a post-operative cast is performed and maintained for 4 weeks. This represents the standard post-operative protocol after any allograft procedure, because allow enough protection from weight bearing and, most of all, from all rotational and torsional stresses at the level of the osteotomies. The impossibility of performing this procedure in the rabbit increased considerably the risk of failure.

With the final modification of the technique, a satisfactory intra-operative fixation has been achieved, with good stability of the allograft and a lower fracture rate. With this technique, however, despite a relatively stable post-operative stability, some late complications (infection and late fracture) occurred and jeopardized the final outcome. In these circumstances, only 5 overall animals were available for analysis, with understandably no room for any statistical consideration.

Another extremely important point to consider is the patency of the vascular bundle inserted into the medullary canal. In this first project, no cells nor growth factors were added to the allograft, and the main study was focused on the implementation of the allograft incorporation by the addition of the vessel. This is a necessary step before moving to the introduction of more complexes procedure and in vitro processes to obtain autologous stem cells. From the macroscopically point of view, at the harvest time it was evident that the vascular bundle was not patent in two out of three cases. As well as macroscopically findings, histology results from Group B showed that the vessels had a full thickness thrombosis, which didn't allow any revascularization of the allograft. There are many reasons that could explain the occurrence of the thrombosis. The extremely small dimension of the vascular pedicle has to be taken into account in first instance; moreover, despite the heparinization of the vessels, their rotation around the major vessels trunk is one of the main possible

reasons for the occlusion. In one case there was no thrombosis evident, and therefore it is possible to transfer the pedicle avoiding the occlusion of the vessels as long as high care is taken to avoid excessive torsion of the pedicle. The choice of leaving as much fat tissue around the pedicle as possible has been made to avoid thrombosis as well. The soft tissues, in fact, protect the small vessels from excessive surgical traumatization and, at the same time, should allow and encourage the formation of artero-venous anastomosis. To improve the outcome of the pedicle patency, an artero-venous anastomosis can be created at the end of the loop; this would improve the blood flow and, moreover, would give the chance to check the flow intra-operatively.

Histologically there was no difference in the graft appearance between the two groups, indicating that the thrombosis most likely occurred at an early stage, avoiding any blood supply. In the only animal in which a satisfactory patency has been seen, different histological results were evident, mainly in terms of endosteal new bone formation. This is encouranging for future studies, as it indicates that improvement in the intra-medullary vascularization in loaded massive allograft might result in higher endosteal bone remodelling, leading to a better allograft regeneration and incorporation. One case however cannot prove this concept, and further studies are necessary to assess this.

Future studies are necessary mainly to improve the animal model. The small animal has demonstrated to be not the best model to test the intra-medullary artero-venous implantation for the improvement of the integration and regeneration of a cortical massive bone allograft, because fixation techniques do not allow a good stability. At the same time, the extremely small dimensions of the vessels require microsurgical techniques to perform an artero-venous anastomosis, with still very high chances of vessel thrombosis. Together with this, the impossibility to perform a post-operative cast to protect the construct further increases the risk of complications. The sheep model, on the other hand, has been proven to be a very reliable and efficient model for intercalary bone graft studies, and we believe that this model will be the one that will give an answer wether a vascular bundle can change the natural history of allograft integration. The metatarsal sheep model not only allow a good and stable fixation with plate and screws, but there is the possibility in the post-operative time, to perform a cast and avoid the weight-bearing, so to improve the chances of allograft integration, exactly as it happens in humans. Unfortunately there are limits with this large animal model: it doesn't allow big number of experimental procedures and it is very costly; moreover, an animal facility with the possibility of working with large animals is necessary. These, however, are limits that can be overtaken by working in a high level experimental facility. The future plan, therefore, is to translate this experiment to the sheep model.

4. SOFT TISSUE REGENERATION PROJECT

4.1 Background

While patients with ESTS were historically treated by amputation (143), current multimodal approaches combining wide surgical resection with (neo)adjuvant radiotherapy and/or chemotherapy allow limb preservation in 90–95% of patients, without compromising disease recurrence and survival (144-148). However, managing tumors with a limb-salvage approach can create complex soft tissue defects susceptible to wound healing difficulties in the postoperative period (105, 149). Reconstructive surgery in the form of pedicled and free tissue transfers has become paramount to the success of limb preservation in these difficult cases. Indeed, surgical flaps help to attain wound closure, fill surgical dead space, protect critical structures (i.e., nerves, tendons, joints), and promote wound healing (105, 149-152).

A very recent study (153) examined the function and health status outcomes following soft tissue reconstruction for limb preservation in extremity soft tissue sarcoma. The primary objectives of this study were: 1) to compare postoperative function and health

status in Extremity Soft Tissue Sarcoma (ESTS) patients undergoing limb salvage with and without soft tissue reconstruction, and 2) to examine the effect of surgical flaps on function and health status at 1–2-year follow-up. Additionally, this study examined the relationship between soft tissue reconstruction and postoperative complications in ESTS patients undergoing limb-preserving surgery. This study has demonstrated that patients who received a flap reconstruction had significantly more impairments and activity limitations at 1–2 years postoperatively than patients in the primary closure group.

A number of factors may contribute to this finding. Patients receiving flaps had significantly larger and higher-grade tumors than patients treated with primary closure, and were more likely to have received preoperative irradiation, bone resection, and motor nerve resection. Each of these tumor and treatment factors has been associated with worse function and/or health status outcomes in at least one prior study of ESTS patients undergoing limb-salvage surgery (154-157). Moreover, patients in the reconstruction group had more preoperative activity limitations. In addition to that, patients receiving flap reconstruction had more complications than patients treated with primary closure in this study (41% of 56 versus 31% of 191), although this difference was not statistically significant. Together with this, having a non-innervated flap may affect the activity limitations and negatively influence the overall outcome. Despite all these findings, interestingly postoperative MSTS and TESS scores in patients requiring extremity reconstruction were not

substantially worse compared to patients who received primary closure. Also interesting is the fact that patients requiring reconstructive surgery in this study reported similar participation and health status outcomes to patients treated with primary closure. This findings suggests that patients with reconstructed extremities are able to maintain similar social and occupational functioning and overall well-being compared with patients not requiring reconstructive surgery, despite greater restrictions in the physical and activity domains of function.

From our prospective, hence, patients who require reconstructive surgery for limb preservation can achieve good functional outcomes. It seems important, therefore, that when a flap reconstruction is necessary in order to guarantee limb sparing surgery, the functional outcome after plastic reconstruction could be improved as much as possible, so to give the best possible chances to these patients to return to their previous activities.

In addition to these considerations, the role of radiotherapy needs to be taken into account. In the mid-1970s the rate of amputation for extremity soft tissue sarcomas was 40–50% (158, 159). Radiation therapy was not considered to be a potentially curative modality for the large tumor masses although palliation of symptoms would often be obtained. In mid 1980s, however, Rosenberg and colleagues reported that when compared with amputation, wide excision with external beam radiation therapy was associated with equivalent 5-year disease-free and overall survival

(160). The combination of surgery and radiotherapy has been proven to yields superior local control compared to local excision alone and has been fundamental to the adoption of limb-conserving therapy for high-risk extremity STS (145, 161-163). Preoperative RT is preferred at our Institute (St Vincent's Hospital, Melbourne) because of smaller RT targets (164, 165), lower RT dose due to better limb perfusion and oxygenation (166) and decreased late toxicity (167) compared with postoperative RT despite a slight higher rate of wound complications.

The role of free or pedicled flaps becomes extremely important after pre-operative radiotherapy, because the damages that the radiations procure to the surround skin do not allow a riskless primary closure (168). The use of re-innervated flaps has proven to be invaluable in numerous reconstructive procedures for example in brachial plexus injuries and Volkmann's contracture or for elbow reconstructions (169), and more recently their use has been proposed in soft tissue sarcomas (70, 170-172).

Re-innervated muscle transfer can provide active contraction and soft tissue coverage, and therefore seems to be extremely suitable for reconstructions after soft tissue sarcomas resection. To our knowledge this is the first study that evaluates the functional outcome of innervated muscle transfer in the setting of irradiated limbs for soft tissue sarcomas in multiple locations and using multiple type of flaps.

4.2 Patients and Methods

From 2006 to 2010, 14 patients have been treated with neo-adjuvant radiotherapy and resection for soft tissue sarcoma followed by reconstruction with innervated flap. The demographic is showed in table 1. The resection and the flap reconstruction were performed in one stage for all the patients. There were 4 malignant fibrohistiocitoma, 4 pleyomorphic soft tissue sarcoma, 3 liposarcoma, 1 neurofibrosarcoma, 1 DFSP and 1 metastatic chondroblastic osteosarcoma with abundant soft tissue extension.

All the patients received pre-operative radiotherapy (range 50-60 Gy) and one received pre-operative chemotherapy associated.

The resection was wide in all the cases except in one, in which the margins were marginal due to pathologic fracture in metastatic chondroblastic osteosarcoma of the femoral shaft with extension in the soft tissues.

The resection involved the lower limb in 11 patients and the upper limb in 3. In the lower leg, the resection involved the posterior compartment in 2 patients (gastrocnemius and soleus in one patient; flexor digitorum longus, flexor hallucis longus and soleus in the other patient) including the tibialis nerve in both cases; in the other one the resection involved the anterior compartment and the muscle replaced was the tibialis anterior.

Pt	Age	Histology	Localization	Tumor Dimensions (mm)	Margins	Adjuvant Therapy
1	40	Dermatofibrosarcoma protuberans	lower leg, post compartment	100 x 55 x 50	wide	RT pre-op
2	68	MFH (recurrent)	Left buttock	260 x 160 x 70	wide	RT pre-op
3	55	Pleyomorphic sarc	Left adductor compartment	250 x 130 x 90	wide	RT pre-op
4	58	Chondroblastic OS (metastatic)	L femur (bone) + antero-medial thigh	130 x 550 x 58	marginal	Ct + RT pre-op, CT post-op
5	75	MFH	L biceps	225 x 150 x 85	wide	RT pre-op
6	83	Pleyomorphic sarc	posterior thigh	245 x 125 x 90	wide	RT pre-op
7	63	Neurofibrosarcoma	popliteal fossa	145 x 75 x 25	wide	Rt pre-op
8	29	MFH (angyomatoid)	medial distal thigh	230 x 105 x 55	wide	RT pre-op
9	70	liposarcoma	L posterior thigh	230 x 130 x 110	wide	RT pre-op
10	67	liposarcoma	upper toracic, scapula	125 x 87 x 30	wide	RT pre-op
11	83	MFH	anterior arm	115 x 100 x 30	wide	RT pre-op
12	67	Pleyomorphic sarc	antero-lateral thigh	350 x 140 x 250	wide	RT pre-op
13	66	liposarcoma	Anterior leg	130 x 65 x 60	wide	RT pre-op
14	64	Pleyomorphic sarc	medial prox thigh	200 x 150 x 120	wide	RT pre-op

Tab. 1 Patients dempgraphics, tumor histology, compartment involved, dimensions of the resection, margins achieved and adjuvant therapy

PT	COMPARTMENT RESECTED	MUSCLE FUNCTION REPLACED	FLAP USED
1	Leg, posterior	Soleus + flexor halluci longus + flexor digitorum longus	Gracilis + sural n (second stage)
2	Hip, extensor	Gluteus max	TRAM
3	Thigh, adductor	Adductors	Gracilis
4	Thight, extensor	Quadriceps	Lat dorsi
5	Arm, anterior	Biceps + brachialis	Gracilis (failed, substituted with lat dorsi)
6	Thigh, posterior	Hamstrings	Lat dorsi
7	Leg, posterior	Soleus + gastrocnemius	Parascapular + sural n.
8	Thigh, extensor	VMO + rectus femoris	Gracilis
9	Thigh, posterior and adductor	Hamstrings + adductor magnus	Lat dorsi
10	Scapular stabilizers	Rhomboid + trapezius	Lat dorsi (pedicled)
11	Arm, anterior	Biceps + brachialis	Gracilis
12	Thigh, antero-lateral	Vastus lateralis + rectus femoris	TRAM
13	Leg, anterior	Tibialis anterior	Gracilis
14	Thigh, adductor	Adductors	Gracilis

Tab. 2 Table showing the compartment involved, the function replaced and the flap used

In the thigh, 1 patient had a femoral resection and reconstruction with megaprosthesis associated with anterior compartment excision; the extensor compartment was excised in other 2 patients. In 2 patients the resection involved the posterior compartment of the thigh, in both cases excluding biceps femoris, but in one including the adductor compartment. In the remaining 3 patients the excision involved the adductor compartment in 2, and the gluteus maximus in the other. In the upper limb, 2 resections involved the anterior compartment of the arm and 1 the rhomboid and trapezius muscles.

The donor flap was selected on the base of the size of the defect to reconstruct and the size of the overlying skin. The overall resection was generally of big dimensions, measuring on average more than 20x15x8 cm (Tab 1), with always a substantial skin resection involved as well as part of the margins. The gracilis myocutaneous flap was used in 7 patients (in 6 innervated, in one the sural nerve was subsequently implanted), the latissimus dorsi in 3 (in 2 cases free, in one pedicled), the TRAM in 2 and the free parascapular with the sural nerve in one (TAB 2).

The vascular pedicles were anastomosed to nutrient vessels available after the tumor resection and the donor motor nerve was sutured to the prepared recipient nerve.

The mean follow up was 16.6 months (range 8-39). The patients were evaluated for: 1) strength in the reconstructed

compartment (MRC scale: Medical Research Council, 1981), 2) range of motion (ROM) of the joint(s) controlled by the muscle(s) replaced, 3) overall function using the Lower Extremity Functional Scale (LEFS) (173) for the lower limb and the DASH (Disabilities of the Arm, Shoulder and Hand) (174) for the upper limb, and 4) MSTS score (175, 176). The evaluation of these scores has been performed by an independent physician blinded from the study. All these scores are presented as percentage, where 100% is the maximum possible score.

4.3 Results

There was one superficial infection at the donor site, treated with oral antibiotics for 7 days. In all the other cases the wound healed with no complications and without any discomfort for the patient.

In terms of disease control, in one case (the patient in which we could not achieve wide margins) there was a local recurrence that led to an above knee amputation. This patient died of the disease and, because the recurrence occurred at less than 12 months post-operatively, the function of the flap was not recorded.

PT	Flap used	Complication	Time of complication occurrence (days)
1	Gracilis + sural nerve (implanted in second stage)	Wound break down	8
2	TRAM	Nil	-
3	Gracilis	Tumor recurrence, died of disease	90
4	Latissimus dorsi	Flap failure (necrosis), substituted with not innervated flap	27
5	Gracilis	Flap failure (necrosis), substituted with re-innervated lat dorsi	5
6	Latissimus dorsi	Lymphoedema	7
7	Parascapular + sural n.	Lymphoedema + superficial infection	11
8	Gracilis	Nil	-
9	Latissimus dorsi	Nil	-
10	Latissimus dorsi (pedicled)	Nil	-
11	Gracilis	Nil	-
12	TRAM	Superficial infection at donor site	5
13	Gracilis	Nil	-
14	Gracilis	Tumor Recurrence (flap evaluated)	420

Tab. 3 Table showing the complications related to the type of flap used, and the time of occurence

Of the remaining 13 patients there were a total of 6 post-operative complications, 2 major and 4 minor (Tab 3). The major complications were flap failures in both cases because of necrosis: in one case the original flap (gracilis) was substituted with another innervated flap (latissimus dorsi); in the other case, the original flap (gracilis) was substituted with a non-innervated VRAM. In both these patients the histology of the removed flap confirmed severe ischaemic damage without signs of infection. Of these 2 patients, only the one with the second attempt of innervated flap has been evaluated for functional results, and the latissimus dorsi was the flap considered for evaluation. The four minor complications were: lymphoedema in 3 (one with superficial infection treated with oral antibiotics) and wound breakdown in one (which required debridement and superficial skin graft coverage). In all these patients with minor complications the original innervated flap has been maintained and therefore subsequently evaluated.

Therefore, a total of 12 patients have been evaluated for functional and emotional results. All of these patients had pre-operative radiotherapy, with a dose of 50 to 60 Gy. The response to radiotherapy was good (>90% of necrosis in the final specimen) in 75% of the cases, and poor (<90% of necrosis) in the remaining 25%. In all but one case the response to radiotherapy was clinically evident, showing a marked decrease of uptake in functional scans (Thallium scans performed at 30 mins and at 4 hours) associated with a

moderate increase in size of the lesion, due to the necrosis of the most inner part of the tumor.

From the functional point of view, in all the patients evaluated there was clinical evidence of re-innervation with some degree of muscle function. All the patients, in fact, were able to move the joint or joints controlled by the muscle replaced. The strength of the muscle (or muscles) replaced was M5 in 7 patients at a mean follow up of 18.1 months (range 8-39) and M4 in 5 patients at a mean follow up of 10.5 months (range 8-14).

The range of motion (ROM) of the joint (or joints) controlled totally or partially by the innervated flap was complete in 7 patients, partial due to muscle weakness in one at an early follow up (8 months), and partial for incomplete re-innervation in one. (TAB 3) Specifically, in one patient the muscle function replaced was the hamstrings, substituted by the latissimus dorsi, and, at a follow up of 8 months, the flexion of the knee was up to 90°, with a strength of 4/5. In the second patient, the compartment replaced was the entire posterior compartment of the leg, involving the flexor hallucis longus, the flexor digitorum, the soleus and the plantaris; in this patient the first reconstruction was with a non-innervated gracilis mio-cutaneous flap, which in a second time was revised and a vascularized sural nerve was implanted to the graft and anastomosed to the tibial nerve. The partial functional result in this particular case consists in full ankle ROM (soleus completely re-innervated) but minimal plantar-

flexion of the toes with inability to stand on them at 39 months of follow up. From the sensation point of view, at 12 months of follow up there was no protective sensation and still numbness at the level of the graft; this slowly improved with, at a final follow up, complete painless sensation.

The overall function was excellent in all the cases. In the three patients in which the reconstruction involved the upper limb the DASH score was 52.2%, 61.4% and 100%. In the lower limb the mean LEFS was 78.4% with a range between 38% and 100%.

In terms of functional associated with emotional evaluation, the MSTS score resulted in a mean of 89.1%, with a range between 43% and 100%.

There was no difference between upper limb and lower limb with regards of functional or emotional scores, even if it has to be considered that the small number of cohorts do not allow any statistical consideration.

PT	Follow up (months)	Muscle Strength	DASH/LEFS	MSTS
1	39	5	86%	90%
2	13	5	100%	100%
3	Died of disease	NA	NA	NA
4	(1)	NA	NA	NA
5	9	4	52.2%	83%
6	11	4	58%	76%
7	14	4	38%	100%
8	14	5	100%	100%
9	8	4	65%	90%
10	8	5	100%	100%
11	9	4	61.4%	43%
12	18	5	71%	100%
13	18	5	97%	100%
14	14	5	91%	86%

Tab. 3 Table showing the complications related to the type of flap used, and the time of occurence

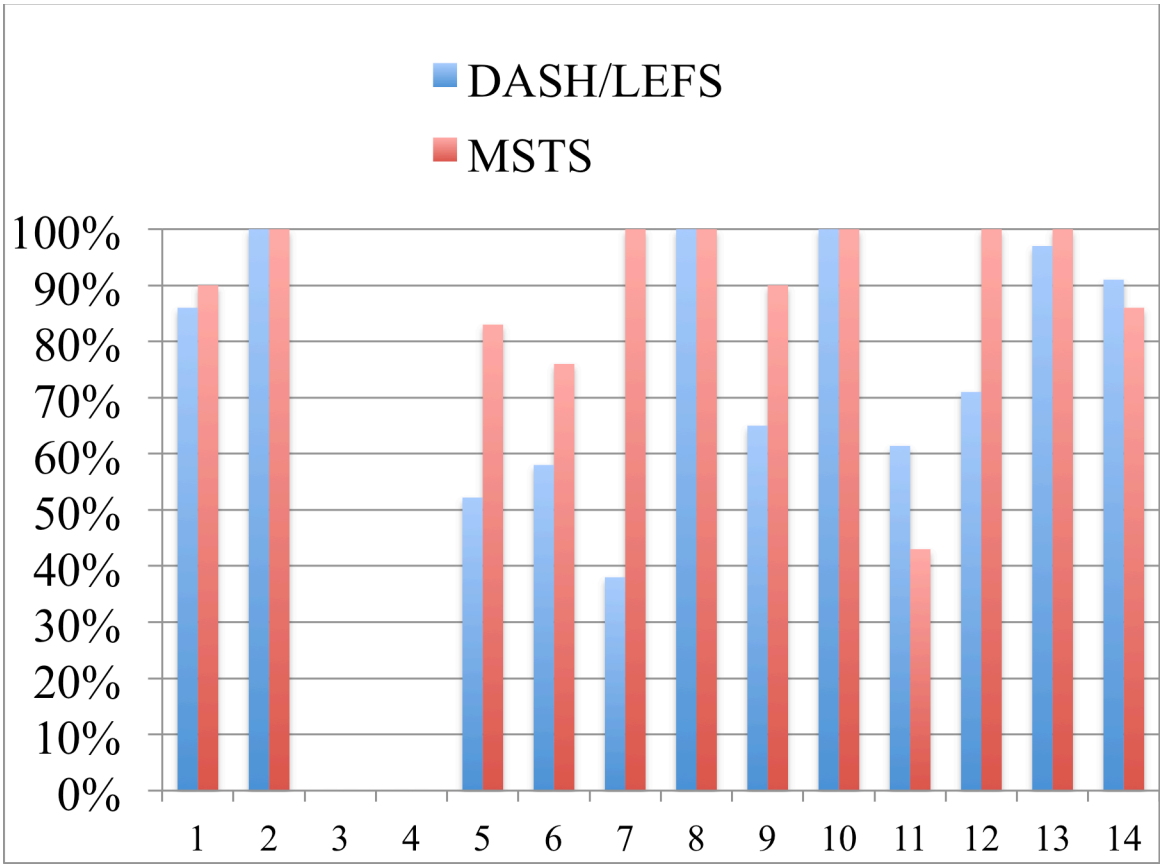

Fig. 24 Functional scores in all the patients; 100% represents the best outcome, with functional results compared to normal. DASH: Upper Limb; LEFS: Lower Limb.

4.4 Discussion

Soft tissue sarcomas are aggressive tumors that require extensive resections to obtain wide margins. Free or pedicled muscle transplantations are often necessary for wound closure, especially when the resection involves a conspicuous amount of muscle and skin. The main goal of plastic reconstruction has traditionally been soft tissue coverage, as it has been seen that in the majority of the cases the remaining muscles are able to become hyperthrophic and to partially replace the function of the resected ones (177, 178). The indication for a functional reconstruction has been limited therefore to the forearm and the posterior leg (178), but more recently this has been extended to the thigh, the anterior lower leg, the shoulder and the buttock (69, 70, 179, 180). In this study we showed that these extended indications are appropriate and that, by providing adequate muscle function after tumor resection, the patient's satisfaction and emotional status can be improved.

It is well known that the use of radiation therapy negatively affects microvascular surgery because it causes intimal damage of the vessels with consequent lower success rate of the anastomosis (62, 181), therefore has been proposed that, when necessary, recipient vessels should be sought outside the field of irradiation to avoid

vascular complication (71). In accordance with this statement, in fact, flap necrosis was seen in 18% of our cases (2/11), but in both cases the flap has been replaced with another muscle transfer and subsequent good result. Despite the negative effect of radiotherapy on flap survival therefore, we showed that it can be possible to perform another reconstructive limb salvage procedure. Moreover, in one of the two patients we were able to implant another re-innervated flap and therefore maintaining the chance of a functional reconstruction.

The complication rate after free or pedicled musculo-cutaneous flap reconstruction is higher compared to primary closure and this is mainly due to the fact that patients receiving flaps have a significantly larger and higher-grade tumors than patients treated with primary closure, and were more likely to have received preoperative irradiation, bone resection, and motor nerve resection (153). Each of these tumor and treatment factors has been associated with worse function and/or health status outcomes (4, 154, 157). In our experience, the use of innervated flaps does not increase the amount or the severity of post-operative complications compared to non-innervated flaps, while providing a possibly better functional outcome. In our experience, the time of the surgery and the amount of blood loss is not influenced by the use of an innervated flap, as shown by other authors as well (70, 71); at the same time, the surgeon experience required to perform an innervated flap is the same

required to perform a free flap, therefore no further training or high learning curve is necessary.

A limitation of this study, together with the small number of cases, is the lack of direct comparison between this technique and the standard not-innervated flaps in terms of functional and emotional outcome. Our study was a preliminary report, which had the goal of the evaluation of the feasibility of this technique and the good functional outcome. In a second time, with a higher number of patients and with a longer follow up, it will be possible to compare the two cohorts and to definitely give an answer to the question whether or not performing an innervated flap is worthwhile. From these initial data, however, the choice of this innovative technique seems reasonable especially when the resection of the tumor involves a substantial amount of an entire compartment. In both the upper and the lower limb the indication is mainly related to the function: in the lower limb the most important functions that need to be maintained are the hip and the knee extension, and the knee flexion. In the upper limb, the important functions that allow the patient to maintain a reasonable quality of life are elbow flexion/extension (prono-supination is usually not impaired if the lesion involves only the soft tissues), and the wrist flexion. In all these cases we believe that the use of re-innervated flaps can give substantial superior results compared to standard reconstruction, associated with a higher satisfaction rate of the patients.

5. CONCLUSIONS

Tumors involving bone and soft tissues are extremely challenging situations, in which the main goal of the surgery is to preserve the life of the patient. With the recent advances of multi-modal treatment, not only the type of surgery has moved from amputation to limb-sparing procedures, but also the survivorship has improved considerably, moving from about 20% at 1 year to more than 70% at 5 years. Although there is a substantial variability between tumor types, the overall increase of the survival rate entails that there is the need to provide better functional outcome to the patients.

Improvement in bone reconstruction involves the field of biological and prosthetic techniques; although in both areas there is large space for innovations, biological reconstructions have by far the higher chances to achieve substantial better outcome. The key factor that needs to be addressed is the re-vascularization of the biological implant in order to obtain a re-vitalized structure. Our study aimed to obtain a direct re-vascularization of a massive allograft in the small

experimental animal. Although the model had limitations and there was an important difficulty in the achievement of a patent implanted vessel in the allograft, we believe that this is a reasonable and promising approach. The future plan in this particular case will be to move onto the large animal, such as sheep, using the already well established surgical model, in order to prove the superiority of this technique compared to the standard ones used in clinic.

Tumors that involve the soft tissues are approached with wide resection and pre or post-operative radiotherapy. Even in these cases, the reconstruction that follows the resection is of extreme importance not only from the anatomic, but even for the functional point of view. The need for the coverage of defects left after the tumor excision led to a strong collaboration between the orthopaedic and the plastic team, with a substantial increase in the amount of flap reconstruction after sarcoma surgery. Even in this case, the increase in the survival rate of these patients pushes the surgeon to obtain the best possible functional outcome. In this study we have demonstrated that the use of the innovative technique of motor re-innervated muscular flaps is effective when the resection involved important functional compartments of the upper or lower limb. Although there was no direct comparison between this type of reconstruction and the standard non-innervated reconstruction, e underlined how the patient satisfaction and the overall functional scores were noticeably high.

There is the need for further studies and, at the same time, for a deeper understanding of the biology processes that drive to bone and muscle re-generation and re-vitalization, with consequent re-establishment of function, but the results obtained in these preliminary studies are certainly promising and encouraging.

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